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THE ANTIMALARIAL ACTIVITY OF METACHLORIDINE (2–METANILAMIDO–5–CHLOROPYRIMIDINE) AND OTHER METANILAMIDE DERIVATIVES IN TEST INFECTIONS WITH *PLASMODIUM GALLINACEUM**

Sterling Brackett and Emanuel Waletzky
with the technical assistance of
Margaret Baker, Carrie Ola Hughes and Hazel Williams

Many of the derivatives of sulfanilamide are more active than the parent compound and their importance in chemotherapy is well known. The isomers of sulfanilamide, meta-aminobenzenesulfonamide (metanilamide) and ortho-aminobenzenesulfonamide (orthanilamide) have been reported (Henry, 1943; Nitti, Bovet and Depierre, 1937; Feinstone, Williams and Florestano, 1942; Marshall, Litchfield and White, 1942) to have no chemotherapeutic action and so far as we have been able to determine, derivatives of these two compounds have not been extensively investigated. The present paper reports the results of the antimalarial trials of a number of metanilamide derivatives prepared in these laboratories (Table 1, compounds marked S in column 3) and in the laboratories of the Calco Chemical Division (Table 1, compounds marked C). A number of the compounds were found to have a high degree of activity both in preventing mosquito-induced and suppressing blood-induced infections of the malarial parasite, *Plasmodium gallinaceum,* in the chicken.

METHODS

The procedures used for determining the relative activities of the test compounds have been described in "A Survey of Antimalarial Drugs, 1941–1945." The accuracy of these determinations is approximately twofold. In brief, the drugs were administered by the drug-diet procedure and the light schedule consisted of alternating three-hour light and dark periods. Sporozoite-induced infections were produced by the intravenous inoculation of the pooled, chilled material, each bird receiving sporozoites from about one-tenth of a mosquito. Treatment was started two days before inoculation and usually continued for ten days. Activity is expressed as the mean prolongation of the incubation period or the proportion of test birds completely protected from the infection. Sulfadiazine was used as the drug standard since it is the most commonly known drug which is prophylactic in *P.*

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* From the Chemotherapy Division of the Research Laboratories of the American Cyanamid Company, Stamford, Connecticut.

gallinaceum malaria. However, as will be seen, the dosage response curve for the metanilamides is different in slope from that of the sulfanilamides.

Blood-induced infections were produced by the intravenous injection of about 300 million parasitized erythrocytes per kilogram of bird one day after treatment was begun. Treatment was continued for four days after inoculation and at that time the parasitemia of each test group was compared with that of the controls and expressed as a percentage of the controls. Quinine was used as the drug standard in blood-induced infections.

To determine whether an infection had been completely prevented or eradicated the test birds were splenectomized since our previous experience with latent infections indicated that a microscopically demonstrable parasitemia invariably develops six to eight days after splenectomy. Splenectomy was performed on birds which remained negative for twenty-one days following the cessation of treatment and these birds were examined for infection by means of blood smears taken on alternate days for fifteen days after splenectomy.

The analytical procedure for sulfanilamides (Bratton and Marshall, 1939) was used, with certain modifications, for determining the blood concentrations of the metanilamides.[1] Usually a single pooled sample was obtained from each group of five birds 4 to 5 days after drug treatment was started and at the end of a dark period. To overcome the tendency of the coupled metanilamides to precipitate, the concentration of the drug in the final dilution was kept below 0.2 mg/100 ml. The addition of alcohol will accomplish the same thing. Since the metanilamides give somewhat less color than the sulfanilamides the amount of blood taken from each bird was increased from 0.02 ml to 0.1 ml when low concentrations of the drug were expected. This results in a 1 : 40 dilution when the samples from the five birds in a group are pooled and made up to 20 cc with water and reagents. Readings were made with a General Electric recording spectrophotometer which permits the detection of relatively small amounts of color. The more active metanilamides produce high activity at blood concentrations below 1 mg/100 ml but unfortunately the analytical procedure gives only approximate values in this region. A limited number of analyses of solutions of known concentration (containing normal blood) indicate that readings between 0.2 and 0.5 mg/100 ml are in general accurate within a twofold range. Since normal blood sometimes gives a reading as high as 0.15 mg/100 ml, readings of 0.2 mg/100 ml are open to question although readings of 0.3 mg/100 ml are probably different from those of untreated bloods.

RESULTS

A. *General, The Metanilamides as a Group*

Several N^1-heterocyclic substituted metanilamides were found to be more active than sulfadiazine and quinine (Table 1). The first twenty-one compounds in this table are given in the descending order of their activity in sporozoite-induced infections as determined on the basis of the drug concentration in the blood. The remaining compounds failed to show activity in our tests and are arranged roughly according to chemical configuration. The quinine equivalent was obtained from tests using blood-induced infections while the diazine equivalents were obtained

[1] We are indebted to Miss Dorothea Babbitt who devised these modifications and made a number of the analyses.

TABLE 1.—*Relative antimalarial activity of derivatives of metanilamide*

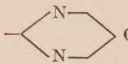
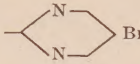
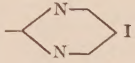
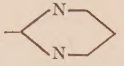
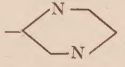
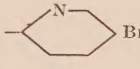
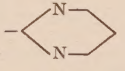
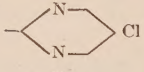
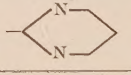
No.	SN* No.	Source† and reference to synthesis	Substitutions in the basic structure				Blood conc.‡		No. of birds	Approximate quinine equivalent§	Approximate diazine equivalent§		Approximate diazine equivalent‖ of p-amino isomer	
			R^5	R^6	R	R′	0.5%	0.1%			Dosage	Blood	Dosage	Blood
A. *Active Compounds*														
1	11,437	S,13	H	H		H	...	1.7	104	16	16	6	8	2
2	12,653	S,13	H	H		H	8	1.0	25	....	16	4	4	2
3	.....	S,13	H	H		H	...	..	10	....	16	....	....	...
4	11,435	S,13	H	H		H	4	1.0	153	2	2	3	1	1
5	11,436	S,13	H	H		H	2.3	0.6	32	0.25	0.75	1	2	1
6	12,203	S,13	H	H		H	1.0	0.4	14	....	0.1	0.75	0.1	...
7	.....	S,13	H	– CL		H	...	0.6	4	....	0.25	ca. 1	1.0	1.0
8	12,658	S,13	H	H		CH_3	...	1.0	9	....	0.25	<1	....	...
9	12,659	S,13	H	H		CH_3	...	0.2¶	10	....	0.1	1	0.05	...

TABLE 1.—(*Continued*)

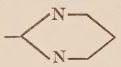
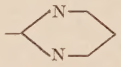
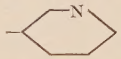
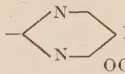
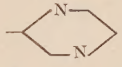
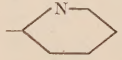
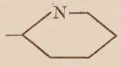
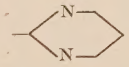
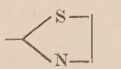
No.	SN* No.	Source† and reference to synthesis	Substitutions in the basic structure				Blood conc.‡		No. of birds	Approximate quinine equivalent§	Approximate diazine equivalent§		Approximate diazine equivalent‖ of p-amino isomer	
			R^5	R^6	R	R′	0.5%	0.1%			Dosage	Blood	Dosage	Blood
A. *Active Compounds (continued)*														
10	12,192	C,14	H	CH_3		H	8	0.7	35	....	0.2	0.5	....	...
11	12,194	C,14	H	CH_3		H	10	0.9	66	0.1	0.6	0.5	....	...
12	12,652	S,13	H	H		H	3	1.0	10	....	0.15	0.5	0.15	0.3
13	12,622	S,13	H	H		H	...	2.0	5	....	0.1	0.5	0.1 (as the Cl analogue)	0.3
14	14,633	S,13	H	H		H	...	..	10	0.02i	0.5	....	....	...
15	12,184	S,13	H	H		H	0.3	..	19	0.02i	0.05	0.3	0.02	0.3
16	12,649	S,13	H	H		H	5.0	0.8	12	....	0.15	0.25	0.15	...
17	7,514	C,14	H	CH_3		H	1.8	0.8	82	....	0.2	0.25	0.3	1
18	12,201	S,13	H	H		H	2.6	1.0	21	0.05	0.1	0.2	0.25	0.6
19	12,182	C,15	H	H	$-COCH_3$	H	3.1	..	9	0.02	0.02	0.1	....	...

TABLE 1.—(*Continued*)

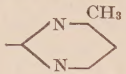
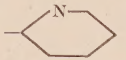
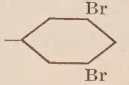
No.	SN* No.	Source† and reference to synthesis	Substitutions in the basic structure				Blood conc.‡		No. of birds	Approximate quinine equivalent§	Approximate diazine equivalent§		Approximate diazine equivalent‖ of p-amino isomer	
			R^5	R^6	R	R′	0.5%	0.1%			Dosage	Blood	Dosage	Blood
A. *Active Compounds (continued)*														
20	12,202	S,13	H	H		H	5.0	1.0	14	0.02	0.02	0.05	0.3	1.0
21	12,204	C,16	H	CH_3		H	0.1	..	7	....	0.01	ca 0.3	....	...
B. *Inactive Compounds*														
22	196	C,21	H	H	H	H	12.6	0.8	14	....	0.02i	....	0.2	0.2
23	12,651	S,13	H	H	$-C(=NH)-NH_2$	H	7.0	1.0	13	....	0.02i	0.02i	0.2	0.5
24	12,654	S,13	H	H	$-C_2H_5$	H	1.0	..	4	....	0.02i	0.1i	....	...
25	.....	C,17	H	H	$-CH_2CH_2OH$	H	4.1	..	4	....	0.03i	0.15i	....	...
26	12,655	S,13	H	H	C_2H_5	C_2H_5	1.0	..	4	....	0.02i	0.1i	....	...
27	.....	C,15	H	H	$-C(=O)-(CH_2)_{12}CH_3$	H	0.0	..	4	....	0.03i	....	....	...
28	12,661	S,22	H	H		....	0.6	..	3	....	0.02i	0.5i	....	...
29	12,646	S,23	H	OH		....	...	..	10	....	0.02i	....	....	...
30	.....	C,14	H	H		....	0.6	..	9	0.02	0.02i	....	....	...
31	234	C,18	H	H		....	2.4	..	5	....	0.02i	0.2i	....	...

TABLE 1.—(*Continued*)

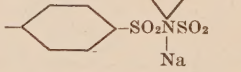
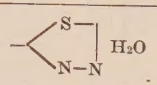
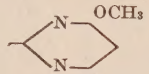
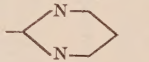
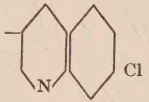
No.	SN* No.	Source† and reference to synthesis	Substitutions in the basic structure				Blood conc.‡		No. of birds	Approximate quinine equivalent§	Approximate diazine equivalent§		Approximate diazine equivalent‖ of p-amino isomer	
			R^5	R^6	R	R′	0.5%	0.1%			Dosage	Blood	Dosage	Blood
B. *Inactive Compounds (continued)*														
32	231	C,19	H	H		H	3.1	..	5	....	0.02i	0.3i	0.1	0.25
33	11,285	C,20	H	H		H	0.0	..	5	....	0.02i	....	....	...
34	14,804	S,13	H	H		....	...	..	5	....	0.02i	....	....	...
35	.....	C,19	H	H		....	2.9	..	5	....	0.2i	0.15i	....	...
36	.....	C,19	H	H		H	0.0	..	4	....	0.02i	....	....	...
37	7,546	C,16	H	CH_3		H	9.1	6.4	15	....	0.01i	0.1i	....	...
38	12,938	S,13	H	OH		H	...	..	5	....	0.02i	....	....	...

TABLE 1.—(*Continued*)

No.	SN* No.	Source† and reference to synthesis	Substitutions in the basic structure				Blood conc.‡		No. of birds	Approximate quinine equivalent§	Approximate diazine equivalent§		Approximate diazine equivalent‖ of p-amino isomer	
			R^5	R^6	R	R′	0.5%	0.1%			Dosage	Blood	Dosage	Blood
B. *Inactive Compounds (continued)*														
39	.....	S,13	H	H		H	0.3	0.0	11	....	0.02i	1.0i	0.05	0.5
40	12,657	S,13	H	H		H	...	1.0	9	....	0.1i	0.1i	0.1i	0.5i
41	14,030	S,13	NH_2	H		....	...	..	15	....	0.05i	....	....	...
42	12,901	S,13	H	H		....	...	..	19	....	0.02i	....	....	...
43	.....	S,13	...	...		....	...	2.4	5	....	1.0i	....	....	...

* Number assigned to respective compounds in reference number 12. A Survey of Antimalarial Drugs.
† S = Stamford Laboratories; C = Calco Chemical Division, American Cyanamid Company.
‡ Blood concentration determinations made while birds on 0.5% and/or 0.1% drug-diet at beginning of light period.
§ Diazine equivalents given on a dosage and/or blood concentration basis. Differences which are twofold or less may not be significant.
‖ These data taken from reference 12.
¶ These analyses taken while birds on 0.2% drug-diet.

from tests using sporozoite-induced infections. The relative degree of activity in both types of infections seems to be fairly parallel. The most active members of the series are 2-metanilamido-5-chloropyrimidine (#1), which has been named metachloridine, and its bromine and iodine analogues (#2 and #3). These compounds are at least sixteen times as active as sulfadiazine and quinine on a dosage basis and six times as active as sulfadiazine on a blood concentration basis.

Earlier reports (Coatney and Cooper, 1944) and our own experience show that, unlike quinine or atebrin, certain sulfanilamides are capable of completely protecting chickens from sporozoite-induced infections of *Plasmodium gallinaceum*. The more active metanilamides, namely numbers 1, 2, 3, 4, 5, and 14, can likewise prevent this infection.

A compound which does not regularly prevent sporozoite-induced infections under conditions of our standard test may not necessarily be more effective when administered for a longer period of time, as illustrated by compound #10. This compound protected only a small proportion of birds when administered at 0.5 per cent in the diet for either eight or twenty days.

TABLE 2.—*Antagonism of sulfadiazine and several metanilamides by p-aminobenzoic acid*

Cpd.*	gm/100 gm diet		Dosage ratio	Blood conc. of Cpd. by itself	Activity ratio†
	Cpd.	PAB	PAB : Cpd.	mg/100 ml	Comb. : Test Cpd.
SD	0.1	0.05	1 : 2	2.5	0 : 9‡
	0.1	0.025	1 : 4	2.5	0 : 9‡
	0.1	0.0125	1 : 8	2.5	0 : 9‡
	0.1	0.00156	1 : 64		13 : 15
17	0.25	0.1	1 : 2.5	2.7	8 : 8
	0.25	0.05	1 : 5	2.7	8 : 8
4	0.1	0.1	1 : 1	1.2	14 : 14
	0.05	0.1	2 : 1	0.4	12 : 12
	0.025	0.1	4 : 1	0.3	5 : 11
1	0.1	0.1	1 : 1	1.5	15 : 15

* SD = Sulfadiazine ; other numbers same as in Table 1.
† A ratio of 0 : 9 indicates that the combination of PAB with the drug markedly antagonized the latter's expected action while a ratio of 8 : 8 indicates that the addition of PAB to the drug did not influence its activity.
‡ Treatment for only four days following inoculation. Rest of groups treated eight days after inoculation.

Because of the similarity in structure of these compounds to the sulfanilamides the possibility that they act by the same mechanism was investigated. One of the characteristics of the sulfanilamides is the extent to which their antimalarial activity can be antagonized by small amounts of p-aminobenzoic acid. The metanilamides are not as readily antagonized by this substance (Table 2) which suggests that they have a different mode of action. Further evidence for this is that the metanilamides are not active against a number of organisms which are sensitive to sulfanilamides and are active against at least one parasite that is relatively insensitive to sulfanilamides. Dr. H. J. White has kindly permitted us to present some of the data (Table 3) obtained by his collaborators in these laboratories which shows that some of the metanilamides with the highest antimalarial activity are either inactive or only slightly active against single strains of seven species of bacteria which are sensitive to sulfanilamides.

Compounds 1, 4 and 5 were tested against the avian coccidian, *Eimeria tenella*, by the procedure described by Waletzky and Hughes (1946) and found to be inactive at 0.5 per cent in the diet although the para-amino isomers, i.e., 2-sulfanilamido-5-chloropyrimidine, sulfadiazine, and 2-sulfanilamidopyrazine respectively show a

TABLE 3.—*Comparative bacteriostatic activity of some metanilamides*

Test organism	Compound*						
	Bacteriostatic concentration in mg/100 ml						
	#1	#4	#5	#6	#10	#11	ST‡
Diplococcus pneumoniae (SVI)	>16	>16	>128	64	>32	>128	1
Staphylococcus aureus	>16	>16	>128	....	....	>128	16
Escherichia coli†	>16	>16	64	....	32	128	$\frac{1}{8}$
Eberthella typhosa	>16	>16	>128	>64	>32	>128	4
Salmonella schottmülleri	>16	>16	>128	>64	>32	>128	16
Neisseria gonorrheae	....	....	64	32	....	64	1
Mycobacterium tuberculosis (607)	>16	>16	>128	32	>32	>128	$\frac{1}{8}$

* The numbers are the same as those used in Table 1.
† Tests made in synthetic media; rest in peptone containing media.
‡ Sulfathiazole.

high degree of activity at lower concentrations. In contrast, the metanilamides are highly active against the malarial parasite, *Plasmodium cathemerium,* in the canary (Hughes and Brackett, in press) which is very resistant to sulfanilamides.

It seems quite evident on the basis of the foregoing data that, in spite of the similarity in chemical structure, the metanilamides and sulfanilamides do not possess the same type of biological activity. An attempt was made to antagonize the activity of the metanilamides with meta-aminobenzoic acid on the assumption that there might be a similar relationship to that between para-aminobenzoic acid and the

TABLE 4.—*Antagonism of the antimalarial activity of 2-metanilamidopyrimidine, #4*

Antagonist	Gm antagonist per 100 gm diet*	Dosage of compound #4 in gm/100 gm diet					
		0.1	0.05	0.025	0.0125	0.00625	0
		Prolongation of incubation period in days					
None	...	17	13	11	9	7	0
m-Aminobenzoic acid	0.25†	11	8	5	2	1	0
p-Aminobenzoic acid	0.1†	18	12	5	2	2	0
Benzoic acid	2.0	10	9	6	4	1	0

* Dosages of antagonist used are the maximum which do not have antimalarial activity in themselves.
† These doses give similar blood levels.

sulfanilamides. Although the activity of some of the less active metanilamides was completely antagonized by large amounts of meta-aminobenzoic acid, large amounts of benzoic acid, or of para-aminobenzoic acid also had antagonistic action (Table 4), as did certain inactive metanilamides, #19 and #37. This lack of specificity and the partial antagonism of sulfadiazine activity by similarly large amounts of benzoic acid and meta-aminobenzoic acid (unpublished data) made it improbable that these findings bear any direct relationship to the mode of action of the metanilamides.

The antimalarial activity of the metanilamides is predominantly or exclusively restricted to those compounds with heterocyclic substitutions (Table 5). Nine

TABLE 5.—*Distribution of antimalarial activity in different classes of N^1-substituted metanilamides. The number of compounds in each category indicated*

	Heterocycles				Isocycles	Acyclic	Un-substituted	Total
	Pyrimidines and pyrazines	Pyridines	Thiazoles	Others				
Active	14	5	1	0	0	1	0	21
Inactive	2	0	2	3	9	5	1	22
Total	..	..	..	..	..	..	..	43

isocyclic and five acyclic substituted compounds as well as the one unsubstituted compound (metanilamide itself, #22) are inactive. Since eight of these inactive compounds, including metanilamide, gave blood concentrations of at least 1 mg/100 ml or higher, and the heterocyclic substituents are active at lower blood concentrations, it is concluded tentatively that metanilamide and its acyclic and isocyclic derivatives do not have significant inherent antimalarial activity. The one possible exception to this in Table 5, the acyclic compound #19, is only very slightly, if at all, active since the incubation period was prolonged by only one day and the parasitemia in a blood-induced infection was only reduced to 30 per cent of the controls. In our limited series, the most active compounds have been the pyrimidines. With the exception of halogenation in the 5-position of the pyrimidine ring, other modifications of 2-metanilamidopyrimidine (#4) have not enhanced activity.

The sulfadiazine equivalents of the para-amino isomers of nineteen of the metanilamides considered here are given in Table 1. It is evident that there is some correlation between the equivalents of the isomers, particularly among the heterocyclic derivatives. This is surprising in view of the probable difference in the mechanism of action of the two groups, and it may suggest that specific heterocyclic substituents endow similar pharmacological properties to both series of compounds and that the pharmacological properties in turn determine to a large extent the relative activities of the compounds. The exceptions to the otherwise good correlation between the isomers (cf. compounds 20, 22, 23, and 32, Table 1) prevent the prediction of metanilamide activity from sulfanilamide activity.

It is noteworthy that the 5-halogenated pyrimidine derivatives are superior in both the sulfanilamide and metanilamide series. It has been concluded that such derivatives of sulfanilamide possess two modes of action, the usual type of action plus some additional mode, since they were only incompletely antagonized by para-aminobenzoic acid and since 2-benzenesulfonamido-5-chloropyrimidine is active while 2-benzenesulfonamidopyrimidine is not. This additional mode of action may also be present in the halogenated meta-amino isomers and will be discussed in detail elsewhere.

B. *Special, Metachloridine and Other of the More Active Metanilamides*

The high degree of activity of metachloridine is evident in Tables 6 and 7. As little as 5 to 10 mg/kg/day prevent sporozoite-induced infections in a large propor-

TABLE 6.—*Titration of the antimalarial activity of metachloridine and sulfadiazine in sporozoite-induced infections of* Plasmodium gallinaceum

Compound	Gm drug per 100 gm diet	Drug intake	Blood conc.	Activity†
		mg/kg/day	mg/100 ml	
Metachloridine	0.006	10	0.3	8/10 cp ; 2/10—13d
	0.003	4.5	..*	5/8 cp ; 3/8—13d
	0.0015	2.5	..	20/20—13d
	0.0004	0.6	..	10/10—7d
	0.0001	0.15	..‡	9/9—5d
Sulfadiazine	0.05	80	1.2	3/5 cp ; 2/5—13d
	0.05	72	1.1	5/5—11d
	0.025	37	0.6	4/4—8d
	0.012	17	0.3	4/4—4d
	0.006	9.5	..	5/5—0d

* Below detectable levels.

† cp = completely protected ; d = average days prolongation of incubation period in other birds ; fraction indicates number of birds in each classification.

‡ By a procedure developed by Dr. J. T. Litchfield, Jr., of these laboratories after these trials were made it was shown that a 0.0001% diet gives blood concentrations of about 0.01 mg/100 ml.

TABLE 7.—*Titration of the antimalarial activity of metachloridine and quinine in blood-induced infections (of four-day duration) of* Plasmodium gallinaceum

Compound	Gm drug per 100 gm diet	Drug intake mg/kg/day	Blood conc. mg/100 ml	Activity†
Metachloridine	0.006	10	0.5	< 1
	0.003	5	0.4	< 1
	0.003	4.5	0.4	< 1
	0.0015	2.1	..*	1.25
	0.00075	1.25	..	18
Quinine	0.05	70	..	< 1
	0.025	35	..	< 1
	0.0125	18	..	40

* Below detectable levels.
† $\frac{\text{Parasitemia of test}}{\text{Parasitemia of controls}} \times 100$; five birds in each group.

tion of the birds and 0.2 mg/kg/day prolongs the incubation period markedly. Blood-induced infections in our standard tests are suppressed by 2 mg/kg/day. These smaller doses result in blood concentrations below levels detectable by available procedures; that is, below 0.2 mg/100 ml. One of the striking features of Table 6 is that as the dose is decreased the resulting activity declines to a greater extent with sulfadiazine than with metachloridine, or, to put it another way, a relatively high proportion of activity is retained even with a marked reduction in the dose of metachloridine. One possible explanation for this is that, as a result of the slow excretion of metachloridine (cf. later discussion), effective blood levels are built up even with very small daily doses. Attention is called to this point because of its implications if the compound should be of some value in suppressive therapy of malaria in man.

This same relationship is not as evident in Table 7 possibly because of the shorter period of treatment which did not permit a similar accumulation of the drug in the blood. Incidentally, the dosage-effect relationship seen with the metanilamides complicates the calculation of oral drug standard equivalents. The conservative sulfadiazine equivalent of 16 was obtained by comparing doses of metachloridine of 5 mg/kg/day with sulfadiazine doses of 80 mg/kg/day, both of which generally protected a major proportion of the birds from sporozoite-induced infections. However, if the equivalent is determined by comparing doses that prolong the incubation period about five days; i.e., 17 mg sulfadiazine ÷ 0.15 mg metachloridine, an equivalent of over 100 is obtained.

Metachloridine is effective as a daily oral dose, although this method of administration is somewhat less effective than "continuous" administration by the drug-diet method. In one experiment with sporozoite-induced infections four single daily doses of 15 mg/kg prolonged the incubation period in five birds for an average of nine days while 12 mg/kg/day administered in the diet for a four-day period completely protected two birds and prolonged the incubation period an average of thirteen days in three other birds. The relatively high activity of the drug when administered as a single dose a day may be due to the slow excretion of the compound although other possibilities cannot be excluded. Available analytical procedures do not permit a determination of the time-concentration curve of the drug in the blood with the small doses used in the experiment just described. When larger doses are used it is found that a considerable amount of drug is retained for twenty-four hours or more by the chicken. This was also found by Robinson et al (paper in preparation) to be the case with dogs.

TABLE 8.—*The rate of action of metachloridine against the erythrocytic stages of* Plasmodium gallinaceum

Drug	Dosage	No. of birds	Mean parasite counts on day indicated following start of treatment—in parasites/100 erythrocytes							
	gm/100 gm diet		0 (0 hours)	1 (24 hours)	2 (48 hours)	3	4	5	6	7
None	..	5	4.9	14.6	50.0	134.0	..	..	..	..
Quinine	0.2	5	4.3	4.1	0.2	<0.1	<0.1	..	..	..
Sulfadiazine	0.2	5	5.8	13.6	11.1	5.9	1.9	0.2	<0.1	..
Metachloridine	0.2	4	4.8	12.0	20.0	15.0	5.7	0.7	<0.1	..

As has been pointed out previously, the more active metanilamides are able to completely prevent sporozoite-induced infections. However, even with metachloridine (0.1% in the diet) and compound #4 (0.5% in the diet) this is true only when treatment is started not later than three days after inoculation and continued for seven days. Metachloridine also failed to eradicate blood-induced infections in eight chickens when treatment was started three days after inoculation even in maximum tolerated doses (1 per cent in the diet for five days followed by 0.5 per cent for the next five days with blood concentrations of 30 mg/100 ml). After the third day of a sporozoite infection certain stages of the parasite may be drug resistant, or in tissues not reached by the drug or in tissues highly endowed with a drug antagonist.

In an earlier paper (Brackett, Waletzky and Baker, 1945) it has been shown that sulfadiazine does not bring about complete inhibition of development and multiplication of the erythrocytic stages of *Plasmodium gallinaceum* for several days, as contrasted with quinine which produces effective inhibition of multiplication almost immediately. A similar comparison of metachloridine with quinine and sulfadiazine (Table 8) indicates that it resembles sulfadiazine rather than quinine in respect to its rate of action, and may even be somewhat slower in action.

Metachloridine, the outstanding member of the metanilamide series in our antimalarial tests, seems to be remarkably non-toxic as well. Doses of about 750 mg/kg/day (0.5% drug in the diet) in the chicken do not alter the normal food intake or weight gain. Maximum blood levels resulting from this dose are about 10 mg/100 ml. With higher concentrations of metachloridine in the diet, which produce blood levels of about 30 mg/100 ml, the weight gains of the birds are reduced. Rats, dogs and monkeys have been shown by others in these laboratories (Robinson et al, paper in preparation) to tolerate blood concentrations of 6 to 30 mg/100 ml for long periods without evidences of toxicity. This evidence, combined with the high degree of activity of metachloridine in avian malaria has encouraged its trial in the malarial infections of man and preliminary reports (Alving, personal communication) are encouraging.

The chemotherapeutic activity of the metanilamides is evidently quite specific, having been demonstrated only in the case of several species of malaria to date. Metachloridine was inactive in several other infections when administered by the drug-diet procedure in large doses which resulted in high blood concentrations. The infections tested were *Trypanosoma equiperdum* in the mouse and guinea pig, *Trypanosoma cruzi* in the mouse, *Leishmania donovani* in the hamster and *Schistosoma mansoni* in the mouse. It has already been pointed out that metachloridine is ineffective against the sporozoan parasite, *Eimeria tenella* in the chicken.

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SUMMARY

1. The relative antimalarial activities of metanilamide and 42 of its derivatives

have been determined using sporozoite- and blood-induced infections of *Plasmodium gallinaceum*. The most active members of the series, 2-metanilamido-5-chloropyrimidine (metachloridine) and its bromine and iodine analogues, are sixteen or more times as active as quinine or sulfadiazine.

2. Sporozoite-induced infections are prevented by as little as 5 to 10 mg of metachloridine/kg/day and 0.2 mg/kg/day prolongs the incubation period markedly. Blood-induced infections are suppressed by 2 mg/kg/day. The lower doses result in blood levels of 0.2 mg/100 ml or less.

3. Even with maximum tolerated doses of over 1 gm metachloridine/kg/day (blood levels of about 30 mg/100 ml) established infections are not eradicated.

4. The metanilamides probably do not act by the same mechanism as the sulfanilamides since they are not readily antagonized by para-aminobenzoic acid, and are ineffective against several species of bacteria as well as the coccidian, *Eimeria tenella,* which are sensitive to sulfanilamides. They are also highly active against *Plasmodium cathemerium* which is relatively insensitive to sulfanilamides.

5. The most active members of the metanilamide series are those with N^1-pyrimidine substituents. With the exception of halogenation in the 5-position of the pyrimidine ring, other modifications of 2-metanilamidopyrimidine have not enhanced activity.

6. Metachloridine is not active against *Trypanosoma equiperdum, Trypanosoma cruzi, Leishmania donovani* or *Schistosoma mansoni.*

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THE PREVENTION OF SPOROZOITE-INDUCED INFECTIONS OF *PLASMODIUM CATHEMERIUM* IN THE CANARY BY METACHLORIDINE[1]

CARRIE OLA HUGHES AND STERLING BRACKETT

This brief report is to call attention to the fact that 2-metanilamido-5-chloropyrimidine (metachloridine) will prevent infection of canaries by the sporozoites of *P. cathemerium*. It also further emphasizes the unusual properties of the metanilamides first pointed out by Brackett and Waletzky (1946). Metachloridine and several other compounds (Table 1) were chosen for trial in sporozoite infections of *P. cathemerium* in the canary because they had been shown to have a high degree of activity in preventing similar infections of *P. gallinaceum* in the chicken (Brackett and Waletzky, 1946; Survey of Antimalarials, 1941–1945) and because they were believed to act wholly, or at least in part, by a mechanism different from that of true sulfanilamides (Brackett and Waletzky, 1946; Waletzky and Brackett, paper in preparation) which prevent infections of *P. gallinaceum* (Coatney and Cooper, 1944) but not of *P. cathemerium* (Maier, 1941; H. Beckman, personal communication). The anti-*gallinaceum* activity of these compounds is indicated in Table 1 for ease of reference.

METHODS

The $3H_2$ strain of *P. cathemerium* (obtained from Dr. Harry Beckman) in female canaries was used throughout the study. Infections were produced either by the subcutaneous or intramuscular injection of pooled sporozoite material obtained by grinding infected *Culex pipiens* mosquitoes in a Ten Broeck tissue grinder using normal chicken serum as a diluent, or by the intravenous injection of enough parasitized erythrocytes to produce a detectable parasitemia immediately. Continuous administration of drug was obtained by dissolving it in the drinking water of the birds and by keeping the birds in continuous light. It was necessary to use small quantities of sodium hydroxide to get the drugs into solution. Pooled samples consisting of 0.02 cc of blood from each bird in a group were used for the determination of the drug concentration in the blood by the Bratton-Marshall procedure. The activity of a compound was determined in tests using sporozoite-induced infections by comparing the time required by control and test groups to reach parasitemias of 0.1 to 1.0%. In tests using blood-induced infections activity was determined by comparing the parasitemias (percentage of parasitized erythrocytes based on observations of one thousand erythrocytes) of the control and test groups at prescribed times.

RESULTS

In sporozoite-induced *P. cathemerium* infections both of the metanilamides tested showed striking activity while the sulfanilamide compound had at best only slight activity (Table 1). Infections were prevented in all four of the birds treated with metachloridine while one of the three birds treated with compound #2 was protected.

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[1] From the Chemotherapy Division of the Research Laboratories of the American Cyanamid Company, Stamford, Connecticut.

TABLE 1.—*The relative activity of several compounds in sporozoite infections of* Plasmodium gallinaceum *and* Plasmodium cathemerium

Compound	SN* number	*Plasmodium gallinaceum*†			*Plasmodium cathemerium*				
					Treatment			Incubation period‖	
		Diazine‡ equivalent	Minimum§ prophylactic dose	Blood conc.	Period¶	Dose	Blood conc.	Controls	Test
			mg/kg/day	*mg/100 ml*		*mg/kg/day*	*mg/100 ml*		
1. Metachloridine (2-metanilamido-5-chloropyrimidine)	11,437	6	10	0.3	1 + 11	275	1.7	5/5–7d**	4/4–cp
2. 2-Metanilamidopyrimidine	11,435	2	150	0.8	1 + 11	300	8.8	5/5–7d**	1/3–cp ; 2/3–19d
3. 2-Sulfanilamido-5-bromopyrimidine	8,605	2	30	2.0	0.5 + 4	300	5.3	5/5–8d	5/5–9d

* Numbers used in "Survey of Antimalarials, 1941–1945."
† Data from Brackett and Waletzky, 1946, for compounds 1 and 2, and Survey of Antimalarials, 1941–1945 for Compound 3.
‡ On a blood concentration basis.
§ Treatment for two days prior to and eight days after inoculation in case of drugs 1 and 2 and for 2 days prior to and five days after in case of drug 3
‖ cp = complete prevention of infection ; d = incubation period in days of birds not protected ; fraction indicates proportion of group in each category.
¶ 1 + 11 = one day prior to and 11 days following inoculation.
** Same controls in each case.

TABLE 2.—*The activity of several compounds in blood-induced infections of* Plasmodium gallinaceum *and* Plasmodium cathemerium

Compound	*P. gallinaceum**	*P. cathemerium*							
	Quinine equivalent dosage basis	Treatment				Parasitemia $\frac{\text{test group‡}}{\text{control group}}$ on day of infection			
		Period†	Drug intake	Blood conc.	No. of birds $\frac{\text{test group}}{\text{control group}}$	3	4	5	6
			mg/kg/day	*mg/100 ml*					
1. Metachloridine (2-metanilamido-5-chloropyrimidine)	16	1 + 6	520	1.2	$\frac{3§}{4}$	$\frac{0.4}{10.6}$	$\frac{0.17}{15}$	$\frac{0.03}{25}$	$\frac{0}{16}$
2. 2-Metanilamidopyrimidine	2	1 + 6	740	14.0	$\frac{4§}{4}$	$\frac{0.6}{10.6}$	$\frac{0.2}{15}$	$\frac{0.1}{25}$	$\frac{0}{16}$
3. 2-Sulfanilamido-5-bromopyrimidine	4	1 + 6	ca. 175	4.2	$\frac{5}{5}$	$\frac{1.3}{15}$	$\frac{3.2‖}{31}$	$\frac{3.2‖}{37}$	$\frac{0.1}{45}$

* From Brackett and Waletzky, 1946, for compounds 1 and 2; from "Survey of Antimalarials, 1941–1945," for compound 3.
† Days prior to and following inoculation.
‡ Expressed as a percentage–average of all counts in a group.
§ Same controls in each case.
‖ These averages are high because of the breakthrough of the infection in one bird of the group.

The criteria for concluding that an infection in a bird was prevented were as follows: 1) negative blood smears (alternate days) for 26 days subsequent to the first appearance of parasites in the controls and, 2) failure to produce infections with sub-inoculation. For the sub-inoculations, 0.2 cc of blood was obtained from each of 4 "protected" birds and 0.1 cc from the fifth. The blood from each bird was inoculated intravenously into two normal birds, each bird receiving 0.1 cc (only 1 bird sub-inoculated from the "protected" bird yielding only 0.1 cc blood). Our limited experience has shown that a demonstrable infection develops in about seven days when 0.1 cc of blood from a latent bird is transferred to a normal bird. The sub-inoculated birds were examined on alternate days from the fourth to the eighteenth day after inoculation.

It is evident that metachloridine is superior to 2-metanilamidopyrimidine since considerably lower blood concentrations of the former protected all birds while only one out of three birds treated with the latter was protected. This difference in the relative activity of these two compounds in *P. cathemerium* malaria is consistent with the results in *P. gallinaceum* malaria (Table 1). It is possible that lower doses of metachloridine or shorter periods of treatment would still protect birds from infection by sporozoites of *P. cathemerium,* since such was the case in *P. gallinaceum* malaria (Brackett and Waletzky, 1946). On the other hand, the data for 2-metanilamidopyrimidine in Table 1 suggest that the metanilamides may be less effective against *P. cathemerium* than against *P. gallinaceum* malaria, since blood concentrations of about 8 mg/100 ml of compound #2 did not prevent all infections with the former, while less than 1 mg/100 ml prevented *P. gallinaceum* infections.

It is not possible, on the basis of results in Table 1 to make a satisfactory comparison between the two metanilamides on the one hand and the one sulfanilamide on the other, since the periods of treatment were not the same. Compound #3 is considerably more active in sporozoite-induced *P. gallinaceum* infections than against sporozoite-induced *P. cathemerium* infections.

The three drugs reported here all have unquestionable activity in the suppression of blood-induced infections of *P. cathemerium* (Table 2). With the limited amount of data available it is not possible to draw any definite conclusions concerning the relative activities of the compounds. However, on a blood concentration basis, metachloridine does seem to be more active than compound #3 since the parasitemias were reduced as much as or more with 1.2 mg/100 ml of the former than with 4.2 mg/100 ml of the latter. 2-Sulfanilamido-5-bromopyrimidine seems to be more effective in blood-induced infections than it is in sporozoite infections, although this may simply be due to the length of the period of administration or some similar factor. The activity of this compound in blood-induced infections of *P. cathemerium* in the canary is consistent with the findings of Marshall (Survey of Antimalarials, 1941–1945) in infections of this species in the duck. This activity can perhaps be attributed to the mechanism which is not antagonized by p-aminobenzoic acid (Waletzky and Brackett, paper in preparation) rather than to its sulfanilamide-type mechanism.

The high degree of activity of the metanilamides against infections of *P. cathemerium,* particularly when sporozoite-induced, as contrasted with the sulfanilamides (Maier, 1941; H. Beckman, personal communication) is of significance in strengthening the earlier conclusions (Brackett and Waletzky, 1946) that these drugs are acting by a different mechanism.

Further trials using *P. cathemerium* infections were not carried out by us, since, under a contract with the Office of Scientific Research and Development, Dr. Wendell Gingrich has developed this infection for antimalarial studies. The compounds reported here have been investigated further by him and will be reported at an early date.

SUMMARY

1. Metachloridine (2-metanilamido-5-chloropyrimidine) prevented sporozoite infections of *Plasmodium cathemerium* in four out of four canaries; 2-metanilamidopyrimidine prevented infections in one out of three birds and greatly prolonged the incubation period in the other two; while 2-sulfanilamido-5-bromopyrimidine has at best very slight activity.

2. All of these drugs suppressed blood-induced infections of *Plasmodium cathemerium* in the canary.

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PLASMODIUM CIRCUMFLEXUM (KIKUTH) IN RUFFED GROUSE IN ONTARIO

A. Murray Fallis
Ontario Research Foundation, Toronto, Canada

The discovery, in the ruffed grouse, of a species of *Plasmodium* resembling *P. circumflexum* was reported previously (Fallis, 1945). The results of experiments to establish the identity of this parasite, study its behavior in the grouse, and compare it with another strain of *P. circumflexum* and *P. lophurae* are reported herein. The plasmodium in the grouse in Ontario is apparently a strain of *P. circumflexum* but for the sake of comparison it will be referred to in this paper as the grouse plasmodium.

MATERIALS AND METHODS

The birds used in the study were infected by intraperitoneal injections of citrated or heparinized blood containing the parasites. The plasmodium from the grouse was isolated from an adult bird taken in November, 1944, at Metagama, Ontario. Strains of *P. circumflexum* and *P. lophurae* were supplied through the kindness of Dr. Clay Huff of the University of Chicago. The grouse plasmodium was transferred to grouse, canaries, ducks, and turkeys. The Huff strain of *P. circumflexum* was carried in canaries only. *P. lophurae* was transferred from chickens to canaries, ducks, and a grouse.

COURSE OF INFECTION OF GROUSE PLASMODIUM IN GROUSE

The original transfer of the grouse plasmodium was made into a six-month-old, parasite-free, ruffed grouse which had been reared in captivity. The transfer was made intraperitoneally with citrated blood from the ruffed grouse taken in November, 1944. Subsequent transfers were made to captive grouse which were 4, 6, and 8 weeks old respectively as well as to canaries, ducks and turkeys.

The general course of the infection, as observed in three of the grouse, is shown in Fig. 1. The prepatent period was 8–11 days. One bird carried the infection for 247 days at which time it died from other causes. The other two birds died from other causes a short time after the infection became chronic. The fourth bird died a few days after the parasites appeared in the peripheral blood. The number of parasites transferred in each case was difficult to determine as, with the exception of bird 217 which died before the infection reached a peak, there was only a single parasite in several thousand of the red cells transferred to the different birds. It is probable that grouse No. 148 was given more parasites than the others as it received about 0.5 cc of blood as compared to 0.08 and 0.15 given to the others. No evidence of pathogenicity of the parasite was observed in any of the infected birds.

DEVELOPMENT OF GROUSE PLASMODIUM IN BIRDS OTHER THAN GROUSE

All attempts to transmit the parasite to chickens failed. The parasite was transmitted to two turkeys 8 and 42 days old, but it was observed in the blood for only one and two days, respectively. The prepatent period in each case was 18 days. No

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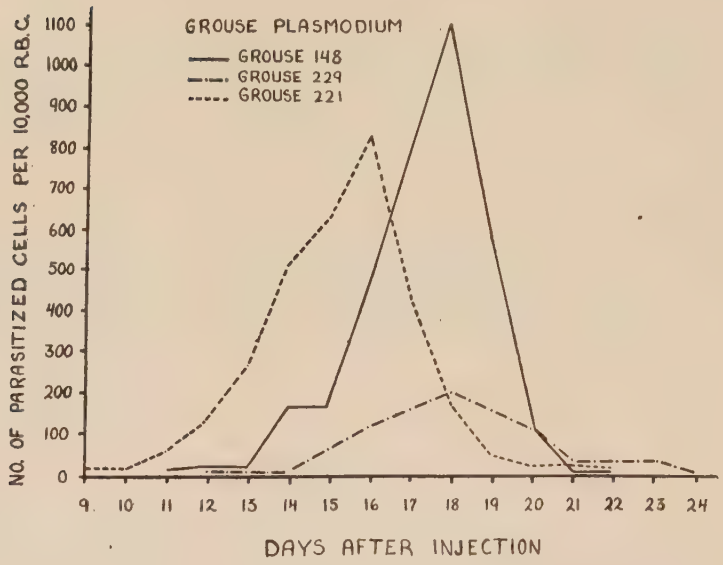

FIG. 1. Course of infection of grouse strain of *Plasmodium circumflexum* in ruffed grouse following intraperitoneal inoculation with blood containing the parasites.

parasites could be found in four other turkeys which received the equivalent or even larger quantities of infected blood. In each of the two positive cases the parasites were scarce with only a single parasite in several thousand cells.

The parasite developed in ducks somewhat more readily than in turkeys, although here too it was observed in only 4 birds out of a total of 19 to which it had been transferred. The birds were 9, 11, 5 and 7 days old, respectively, when infected. In three of these birds the parasite was observed in the peripheral blood for 4 days only and in the fourth for 6 days. The course of the infection in the latter is shown in Fig. 2. The prepatent period was 10, 10, 12, and 18 days, respectively. The length of the prepatent period was not determined solely by the number of parasites

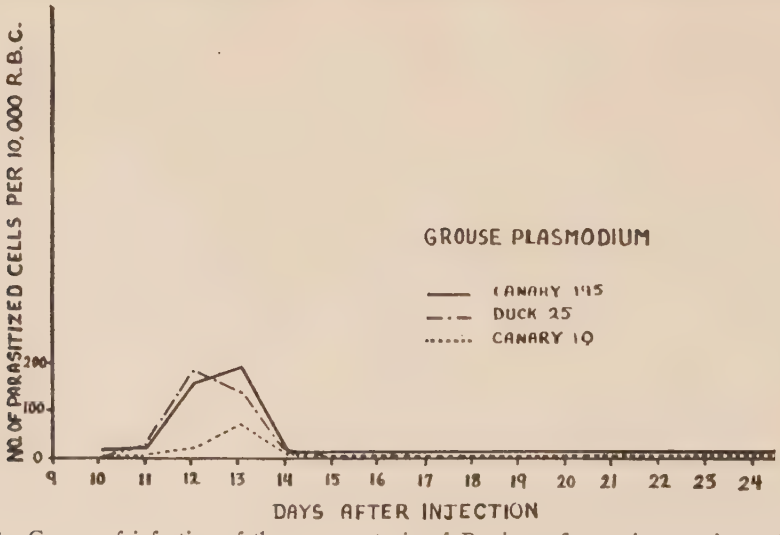

FIG. 2. Course of infection of the grouse strain of *P. circumflexum* in canaries and a duck following intraperitoneal inoculation with blood containing the parasites.

given as the bird with the longest prepatent period received more parasites than any of the others.

Infection with this plasmodium could be given to canaries more readily than to ducks and turkeys although here too development was observed in only about 80% of the 39 birds to which it was transferred. Moreover in many canaries relatively few of the cells were parasitized even at the height of the infection. The course of the infection in a few of the canaries in which it reached its highest levels is shown in Fig. 2.[1] It was observed that, with a few exceptions, the infection was apparent in the canaries for a few days only. It did remain in one bird for 40 days and in two others for 21 and 18 days. The parasite has now been passed from canary to canary through twelve transfers but thus far there has been no significant difference in the duration of the infection in the bird which received the twelfth transfer as compared with the bird which received the first. In some instances canaries received forty to fifty times as many parasites as others and yet no infection was observed in the birds which received the larger doses. It was obvious therefore that factors other than the number of parasites administered could determine whether a particular infection developed in a canary.

Some canaries were given repeated transfers of infected blood. Fewer birds became infected following the second inoculations. It was also observed that the parasites reached a lower level and remained for a shorter time in the peripheral blood of these infected birds.

There was no evidence that the parasite was pathogenic in any of the birds to which it was transferred.

MORPHOLOGY OF THE GROUSE PLASMODIUM

The morphology of the parasite in the grouse was figured in a previous paper (Fallis, 1945). The parasite, although of the elongate type, frequently does not surround the host cell nucleus in the grouse, but does so in the case of the canary red blood cells which are smaller.

The average number of merozoites in schizonts, which appeared to be almost mature in the grouse, was 16 with a maximum of 22 and a minimum of 10.

A distinction in the morphology of the parasite in canaries, as compared with that in grouse, appeared following second and successive transfers from canary to canary. Whereas in the initial transfers of the parasite from grouse to canaries the average number of merozoites in mature schizonts was 16, as in the grouse, with a maximum and minimum of 22 and 9 respectively, in subsequent transfers the average had risen to 19 with a maximum of 26 and minimum of 9 (Fig. 3).

COMPARISON OF GROUSE PLASMODIUM WITH OTHER SPECIES

The morphology and life history of the grouse plasmodium in the grouse and canaries, as observed in the first transfers, suggested that we were dealing with a species with characters midway between *P. lophurae* and *P. circumflexum.* Some experiments were carried on therefore to compare these species with that found in the grouse. The former was transferred to chickens, canaries, a grouse and ducks.

[1] Since this paper was prepared there has been a single instance following the transfer of the parasite from canary to canary in which a parasite level of 1830 parasites per 10,000 red blood cells was reached during the infection. This level is higher than that observed in any of the grouse.

The latter was maintained in canaries. A single attempt was made to transfer the Huff strain of *P. circumflexum* to an eight-month-old ruffed grouse but no apparent infection resulted in spite of the fact that approximately 29×10^9 parasites were administered intraperitoneally. *P. lophurae* developed in an eight-month-old grouse following administration of 2 cc of infected red blood cells from a chicken in which 40 per cent of the cells were parasitized. The course of the infection is shown in Fig. 4. Parasites were still present in the peripheral blood of the grouse at the time of its death, which occurred two months after receiving the infection. The strain

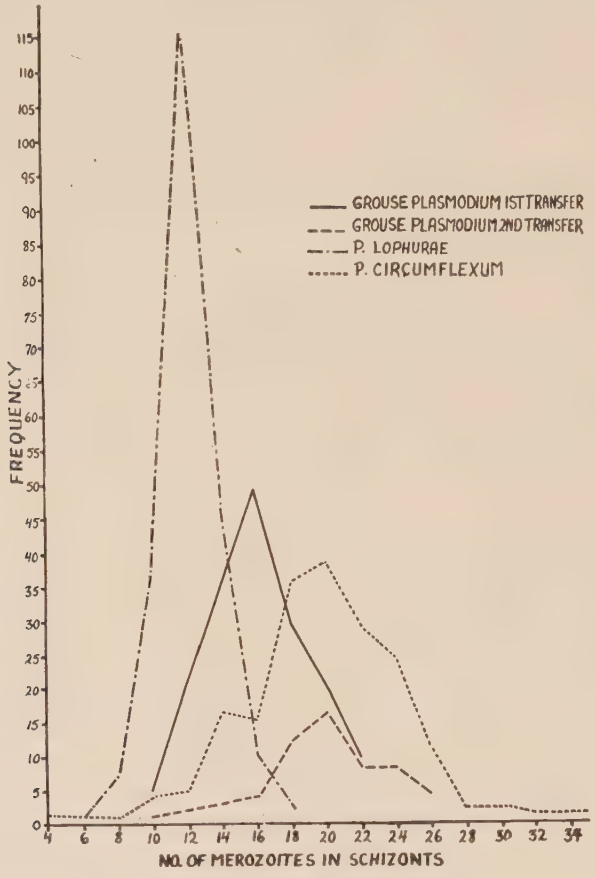

FIG. 3. Frequency diagram of the number of merozoites in mature schizonts of different species and strains of *Plasmodium*.

of *P. circumflexum*, when received from Dr. Huff was pathogenic to most of the canaries to which it was transferred. In later transfers, when fewer parasites were given, they appeared to be less pathogenic and the infection did not reach such a high level. A comparison of one of the first transfers with the 10th (D and W) is shown in Fig. 5. This strain of *P. circumflexum* was short-lived in canaries as compared to some strains which have been described. The grouse plasmodium, as indicated above, showed a similar characteristic when transferred to canaries.

P. lophurae was fatal to young chicks but it seems to me not without significance that this happened only if large numbers of parasites were administered to very young birds. Otherwise the infection was low grade or did not take at all. It

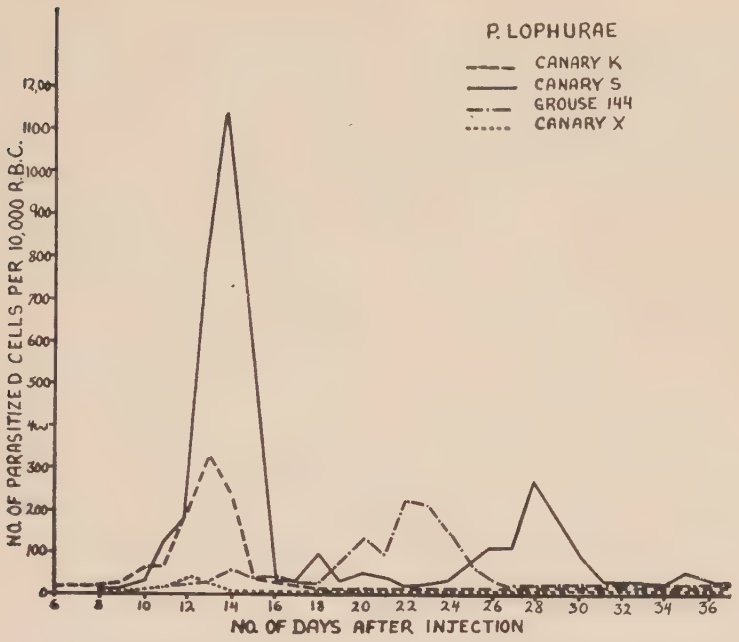

FIG. 4. Course of infection of *P. lophurae* in different birds following intraperitoneal inoculation with blood containing the parasites.

would seem significant also that no difficulty was experienced in transferring *P. lophurae* to canaries and one of the first of such transfers has remained in the canary for eight months without evidence of pathogenic effect. The course of the cycle in some of these canaries is shown in Fig. 4. More variation was shown in the course of the infection in the different birds than was observed for either the Huff strain of *P. circumflexum* or the grouse plasmodium.

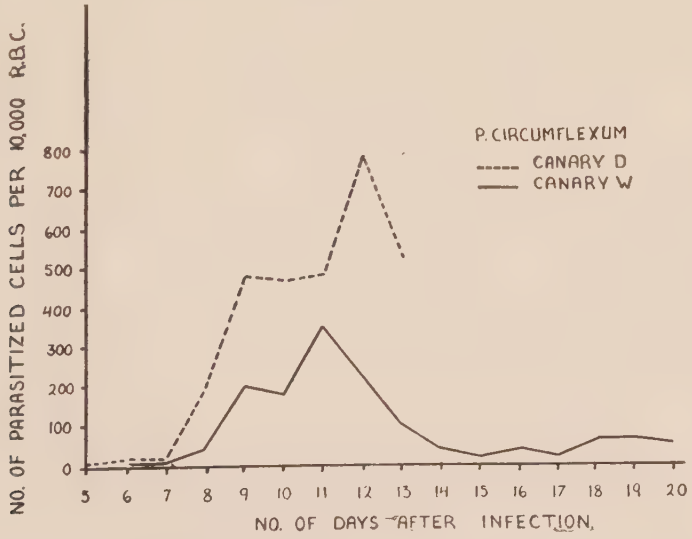

FIG. 5. Course of infection of Huff strain of *P. circumflexum* in canaries as produced by one of the first transfers made during this study (Can. D) and that following the tenth transfer from canary to canary (Can. W).

No development of the grouse plasmodium was observed in six adult pheasants following the intraperitoneal injection of as much as 0.3 cc of canary blood containing 130 parasites per 10,000 red blood cells or in the one instance of 0.34 cc of canary blood containing 850 parasites per 10,000 red blood cells.

A comparison of the number of merozoites in mature schizonts in birds infected with *P. lophurae, P. circumflexum* and the species from the grouse is shown in Fig. 3. The chart includes counts of the merozoites in the schizonts of the grouse plasmodium, in grouse, canaries, and in a duck, following the first transfer to them. Also shown on this chart are counts on schizonts resulting from second and subsequent transfers of the plasmodium to canaries and a duck.

It will be observed that the range in number of merozoites in mature schizonts of *P. circumflexum* extends over that of *P. lophurae* and the grouse plasmodium. Moreover, there is a distinct difference in the average number of merozoites in the schizonts of the grouse plasmodium following the first transfer to other birds as compared with the second and subsequent transfers to canaries and a duck. No such variation was observed in *P. lophurae* infections, however, even up to the fourth successive transfer from canary to canary. This suggests that *P. lophurae* is a distinct species but that the plasmodium in the grouse is a strain of *P. circumflexum*. Some of the *P. lophurae* infections (Can. S, Fig. 4) showed a second rise following the initial one. A similar rise was not observed in infections with *P. circumflexum*. This together with the difference in the number of merozoites in the schizonts of *P. lophurae* are features which help to distinguish it from *P. circumflexum*.

No differences were observed with reference to the gametocytes in the species investigated. The percentage of gametocytes was higher in some birds than others but the cause of this has not been determined.

PERIODICITY

The grouse plasmodium showed little synchronicity or periodicity (Fig. 6) as determined by counting, at 4-hour intervals, the number of uninucleate trophozoites in 100 parasites. It has been found by Wolfson (1936), who counted several growth stages of *P. circumflexum* at least twice daily, that this species has a 48-hour periodicity. The number of uninucleate trophozoites and segmenters was followed during the present study at four-hour intervals in two canaries infected with *P. circumflexum* (Fig. 7). The results suggested a 48-hour cycle in one but a 36-hour cycle in the other. Infection with *P. lophurae* in a grouse was followed in a similar way (Fig. 7). Although no clear-cut periodicity was observed in this infection with *P. lophurae,* there was a suggestion that the asexual cycle may be completed in fewer than 48 hours and perhaps in about 36 hours. From the work of others it would appear that in this species the length of this cycle may be variable. Coggeshall (1938), who described the species, believed the asexual cycle to be about 24 hours. Terzian (1941) thought it was nearer 48 hours, and Hewitt (1942) reported a range of 24–48 hours between the peak abundance of rings or amoeboid forms but stated that in the majority of instances the interval between the peaks was nearer 36 hours. On the basis of this information it might appear that the species in grouse is distinct from *P. circumflexum* and *P. lophurae*. Nevertheless, as all the infections in the present study were established by blood transfer, rather than by sporozoites, it is not surprising perhaps that no clear-cut periodicity was observed in the grouse

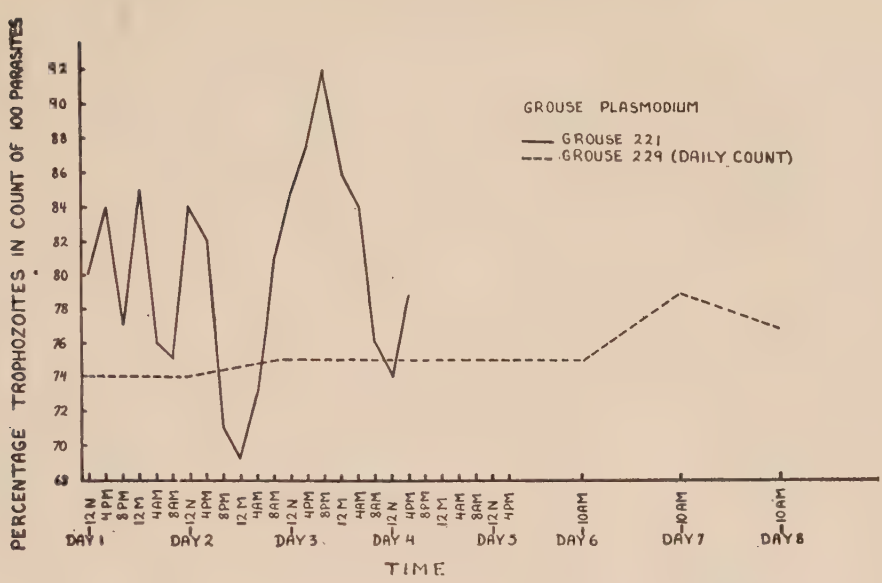

FIG. 6. Percentage of uninucleate trophozoites observed at intervals in peripheral circulation of grouse infected with the grouse strain of *P. circumflexum.*

plasmodium, although it would appear to be rather like a mixture of the above two species. In none of the above species does periodicity seem sufficiently clear cut to be a specific character.

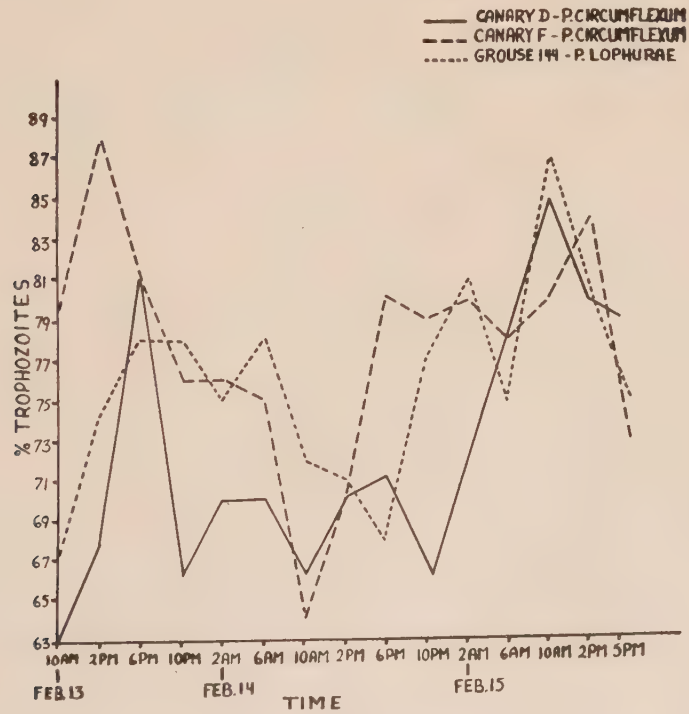

FIG. 7. Percentage of uninucleate trophozoites observed at regular intervals in the peripheral blood of birds infected with the Huff strain of *P. circumflexum* and with *P. lophurae.*

DISCUSSION

The species of *Plasmodium* which has been discovered in grouse appears, on the basis of its morphology and behavior, to be a strain of *P. circumflexum,* although in the grouse itself, and in the first transfer from grouse to canaries, the number of merozoites in mature schizonts would seem to place it mid-way between *P. circumflexum* and *P. lophurae.* The fact that infection did not develop in chickens following administration of infected blood is suggestive of *P. circumflexum.* If tremendous doses of parasites had been given to very young chickens it is possible that the infection might have taken, for Manwell (1933) did succeed in producing low-grade infections in chicks with *P. circumflexum* and more recently Herman and Goldfarb (1939) confirmed his findings. On the other hand the failure to infect a grouse with the Huff strain of *P. circumflexum* was surprising.

It was found in the present investigation that *P. lophurae* could be given to canaries readily, was not pathogenic to them, and may remain chronic in them for several months. It seems to me that although chicks are used to maintain *P. lophurae* they are not normal hosts as the infection can be given to young chicks only when massive doses of infected blood are administered. Moreover, when attempts were made to transfer the *P. lophurae* infections to ducks by giving small quantities of blood only low-grade infections of short duration developed. *P. lophurae* developed readily in the one grouse to which it was transferred but on the other hand the failure of the grouse plasmodium to develop in pheasants suggests that it is not a strain of *P. lophurae.*

The results of these transfers prompt the inquiry as to why ducks have not been found infected in nature if *P. circumflexum* will develop in them, or if they are natural hosts to *P. lophurae.* Yet no species is listed for this bird in Herman's check list (1944). The present study suggests, if small numbers of parasites are transferred, that ducks are no more suitable hosts for *P. lophurae* than they are for *P. circumflexum* and in the case of the latter, as shown by the grouse strain, the infections produced are of low grade and short duration. Consequently even if such infections do occur in nature the chance of finding them is limited.

The duration of the infection in canaries showed almost as much variation in one species as another. The grouse plasmodium was found in peripheral blood of canaries from 1 to 40 days. It remained in the peripheral blood for 14 days following one of the first transfers from grouse but in a number of subsequent transfers it was present for only 1–7 days. It was observed for 40 days in the blood of one bird after the 6th transfer from canary to canary. But following the 7th transfer from this bird to another it was apparent for 6 days only. It was not pathogenic to any of the birds to which it was transferred. The Huff strain of *P. circumflexum* was observed in the peripheral blood from 1 to 25 days. It was pathogenic to canaries following the first transfers; but later transfers did not produce fatal infections. *P. lophurae* remained in canaries from 9 to 257 days and was not pathogenic. It is probable that it would have remained much longer in the case of one bird which died from other causes 257 days after the parasite was first observed in the peripheral blood.

CONCLUSIONS

The plasmodium in grouse is apparently a strain of *P. circumflexum,* which de-

velops more readily in grouse than canaries at least up to twelve successive transfers from canary to canary.

The grouse strain of *P. circumflexum* is distinct from *P. lophurae* although some characteristics are similar.

The strain of *P. circumflexum* in the grouse is apparently not pathogenic, at least when the infection is produced by the transfer of asexual parasites. It is probably not a factor, therefore, in causing periodic dying off of the ruffed grouse in Ontario.

ACKNOWLEDGMENTS

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MIGRATION OF *ENDAMOEBA HISTOLYTICA* ON SOLID MEDIA*

THOMAS L. SNYDER AND HENRY E. MELENEY
Department of Preventive Medicine, College of Medicine, New York University

Although amoeboid cells can accomplish progressive movement only on solid surfaces, they need not be confined, like freely swimming forms, below the surface of a fluid menstruum. Not only distinctly protozoan organisms, such as *Hartmanella* (Shinn and Hadley, 1936) and various soil amoebae (Mouton, 1902; Musgrave and Clegg, 1905) and marine amoebae (Rice, 1934), but even the myxamoebae of "slime-molds" (Raper and Smith, 1939) are capable of moving over the very slightly moist surfaces of 1.5 to 2.0 per cent agar gels exposed directly to the air. However, locomotion under these conditions has not been reported in the case of *Endamoeba histolytica*. While this amoeba is usually cultivated on slants of solid medium (agar or coagulated egg), the addition of a fluid to cover the slant appears to be essential. Since, in view of the reports noted above, this covering fluid can hardly be necessary for amoeboid movement *per se*, it seemed likely to us that its purpose is to satisfy, through the action of the bacteria growing profusely therein, the requirement on the part of *E. histolytica* for an anaerobic environment (Snyder and Meleney, 1943). If this is the case, it should be possible to cultivate this amoeba on solid media and to observe normal locomotion under anaerobic conditions without a covering fluid. Such observations are described below.

The ability of *E. histolytica* to move over the free surface of a solid medium introduces the possibility of developing a migration method for separating the amoebae from their accompanying bacteria. Since first described by Beijerinck (1896, 1897), this method has frequently been used with amoeboid forms to replace the normal bacterial flora by selected species. We have carried out a few preliminary experiments on the purification of *E. histolytica* by migration without success. Since we do not expect to have an opportunity to continue these studies, we are presenting our results in the hope that they may suggest a more fruitful approach to other investigators.

The simplest medium on which abundant growth and migration of *E. histolytica* were obtained had the following composition:

Whole horse serum	25.0 per cent
Difco yeast autolysate	2.5 " "
Difco proteose peptone	0.5 " "
Bacto agar	1.5 " "
Ringer's solution*	70.5 " "
Cholesterol	1 microgram/ml

* The modification of Ringer's solution used in these studies consisted of 0.7 per cent sodium chloride, 0.025 per cent potassium chloride and 0.03 per cent calcium chloride in distilled water.

The hydrogen-ion concentration was adjusted to pH 6.8 to 7.2 with sodium hydroxide. After autoclaving 15 minutes at 121° C, the medium was dispensed aseptically into 16 × 150 mm culture tubes, four ml per tube, and degassed at a pressure

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of 10 to 20 mm of mercury while still fluid. After solidification the slants were stored overnight in an evacuated jar at room temperature. Any fluid which had accumulated at the butt of the slant was removed with a sterile capillary pipette and the surface of the medium dusted lightly with the sterile fine rice starch preparation described previously (Snyder and Meleney, 1943). The slants were inoculated by introducing at the butt one drop of stock culture of *E. histolytica* and accompanying bacteria, grown on coagulated egg slants covered with Ringer's solution containing rice flour. Comparable results were obtained with the Dobell (1931) monkey strain (NRS) and a more recently isolated strain (P). This inoculum, together with additional fluid which appeared during incubation at 37° C, contributed about 0.1 ml of liquid which apparently could not be avoided. The extent of migration was always measured from the meniscus of this fluid. The cultures were maintained in a vertical position and carefully handled in order to prevent wetting more than the lowest 5 to 10 mm of the slant surface.

Anaerobic conditions were provided by two methods. In the first of these, each tube was closed with a rubber stopper lubricated with glycerol and bearing an exhaust tube and clamp, and was evacuated to a pressure of about 110 mm of mercury with a Hyvac pump. In the second method, oxygen was absorbed and carbon dioxide produced by a mixture of 0.4 ml 20 per cent pyrogallol and 0.7 ml 20 per cent sodium carbonate placed on a cotton sponge in each tube, which was then closed with a solid rubber stopper. With both methods, the original cotton plug was retained in the tube, having been pushed down near the slant before producing anaerobiosis. No precise determination of the degree of anaerobiosis attained by either method was made. Methylene blue incorporated in the medium was rapidly reduced and, except on rare occasions, did not become reoxidized. It seems probable that the bacteria present consumed any oxygen introduced by slow leakage of air.

Cultures were examined *in situ* with 16 and 8 mm objectives focused through the wall of the culture tube which was carried on a special mechanical stage graduated in mm and attached to a horizontally inclined microscope. It was found necessary for the line of vision to pass through the agar to the surface of the slant, since droplets of condensed water on the free inner wall prevented examination from that side. A coverslip attached to the tube with a drop of water or oil increased the depth at which an image could be obtained, but even with this aid approximately the lowest one-fifth of the slant surface could not be brought into sharp focus.

Under these conditions actively motile trophozoites could usually be observed on the free surface of the slant after about 24 hours of incubation. They were found extending 10 to 20 mm above the meniscus of inoculum and condensate at this time, reached a height of 20 to 40 mm within 48 hours, and frequently covered the entire slant (about 60 mm) in three to five days. They varied in number from a few individuals per microscopic field to a practically contiguous layer one cell deep.

Bacterial growth could always be observed 4 to 12 mm below the advance rank of amoebae. Since a high magnification could not be used, the apparent absence of bacteria in the foremost zone may have been misleading. The continued extension of both amoebae and bacteria during the incubation period suggests that the bacteria were, in fact, present in this area, having been spread mechanically by the movement of the trophozoites. Such a sequence of events has been described in the case of other amoebae (Mouton, 1902; Musgrave and Clegg, 1905; Shinn and Hadley,

1936). However, the fact that the advance of *E. histolytica,* which is capable of moving at a rate of several mm per hour, was restrained to the neighborhood of profuse bacterial growth indicates that only this region was suitable for migration. In one case a streak of bacteria was inadvertently inoculated along the surface of the slant and it was noted that the trophozoites extended a considerable distance along the sides of this streak as early as 48 hours. Other amoebae have shown a similar preference for the area adjacent to bacterial growth. Mouton (1902) observed that a soil amoeba migrated more rapidly along streaks of bacteria than over uncontaminated agar, and *Hartmanella castellanii* was found to encyst on agar plates if it did not encounter colonies of suitable bacteria (Shinn and Hadley, 1936). No cysts were noted in our cultures. Like Mouton's soil amoeba, *E. histolytica* appeared larger in size when found in an area apparently free of bacteria. After several days of incubation, the amoebae near the butt of the slant were small, inactive and few in number.

We have investigated the effect of a few modifications of the medium on growth and migration of the amoebae. The initial hydrogen-ion concentration had no definite effect within the range pH 5.5 to 7.0. Serum was essential under our experimental conditions. The soluble carbohydrates, glucose, maltose or dextrin, had no appreciable influence on growth or migration, but so increased gas production and disruption of the agar as to interfere with observation. The addition of human erythrocytes, yeast cells (*Saccharomyces cerevisiae*) or heat-killed bacteria (mixed flora from stock *E. histolytica* cultures) to the surface of the slants did not stimulate growth or migration. Ingestion of yeast cells, but not of erythrocytes, was noted when trophozoites were removed and examined on slides.

The omission of starch did not prevent growth or migration, although both were appreciably decreased. It was only in the absence of starch, however, that the amoebae could be observed with the naked eye, in which case they provided the agar surface with a ground glass appearance strikingly similar to Mouton's description (1902), *"elles ne donnent qu'on léger dépoli, une apparance mate à la surface, partout ailleurs brilliante, du milieu de culture."*

In a single culture we observed paths or trails made on the surface of starch-free slants by the passage of *E. histolytica.* These paths were approximately the width of the moving amoeba and resembled scratches on the agar rather than bacterial growth. Their distribution suggested considerable movement by a few individuals. No explanation of their nature is evident since they were not consistently found under any particular experimental condition. An apparently similar phenomenon is described as characteristic of *Flabellula mira* (Rice, 1934) and an occasional occurrence with soil amoebae (Mouton, 1902).

Our comparison of various methods of producing anaerobiosis was probably hampered by the presence of accompanying bacteria. The results described below suggest that a decreased oxygen tension and an elevated carbon dioxide tension are essential for growth and migration of *E. histolytica.* Since the bacteria presumably perform both of these functions to some extent, it was not surprising to find that growth and migration occurred after a delay of several days in tubes merely closed with rubber stoppers. In this case, however, the trophozoites did not extend beyond clearly recognizable bacterial growth. No amoebae were observed in tubes closed with cotton plugs alone. In order to obtain migration during the first two days of

incubation, additional agencies for reduction of the oxygen tension were required. The reduced pressure method appeared to favor migration rather than growth (i.e., fewer amoebae extended over a larger area) at pressures below about 80 mm of mercury. At pressures above 180 mm the amoebae were confined to the bacterial growth, which, in turn, was restricted to within about 12 mm of the fluid meniscus until after four to eight days of incubation. Therefore, it was concluded that a relatively low oxygen tension is necessary in order to induce the amoebae to advance appreciably ahead of the bacteria. The smaller number of amoebae found at very low pressures may possibly indicate the importance of carbon dioxide for growth of *E. histolytica* as opposed to locomotion, since low oxygen tensions would decrease bacterial production of carbon dioxide. The influence of this gas was also indicated with the pyrogallol anaerobic method, since amoebae were found only once in several experiments in which the pyrogallol was alkalinized with sodium hydroxide, whereas they were consistently observed when sodium carbonate was used. An attempt was made to determine the most suitable ratio of sodium carbonate and pyrogallol. The results were too variable to define an optimum range, but the proportion recommended by Foley (1942), seven parts 20 per cent sodium carbonate and four parts of 20 per cent pyrogallol, was uniformly satisfactory.

A few successful transfers were made from slant to slant. The apparently bacteria-free area of amoebic migration was marked on the outside of the tube during examination with the microscope, then a block of agar was taken from between the marks with a stiff platinum loop and inverted on the center of a sterile slant. The pyrogallol-sodium carbonate anaerobic method was used. Three consecutive transfers had been effected without difficulty by the time these studies were terminated. Both amoebae and bacteria could be observed after incubation of each transfer, but when the third subculture had been attained the amoebae showed neither abundant growth nor extensive spread beyond the block of agar transferred. This series demonstrated the possibility of transferring trophozoites, although these forms have previously been shown to be sensitive to air (Snyder and Meleney, 1943). The results were not promising, however, with respect to the problem of separating *E. histolytica* from bacteria. Although some difficulty was experienced in selecting material from the relatively narrow, apparently bacteria-free zone, it appears more likely that failure was due to the presence of small numbers of bacteria in this area. Nor is it at all certain, considering the bacteriophilic tendency of the amoebae under our experimental conditions, that growth or even survival of *E. histolytica* would have been obtained had separation been effected. The success of this method will probably depend upon the discovery of conditions which permit much more rapid and extensive migration of the amoebae beyond the area of bacterial growth.

SUMMARY

1. Conditions are described which permit growth and migration of *Endamoeba histolytica* on the surface of solid media without a covering fluid.

2. Under optimum conditions the trophozoites may be seen migrating beyond visible bacterial growth within 24 hours, and usually extend over the whole slant within a few days. The migration of trophozoites is followed by advancing bacterial growth, with a relatively narrow margin. The amoebae are apparently restricted to an area of medium covered by, or adjacent to, profuse bacterial growth.

3. Indirect evidence indicates that marked reduction of the oxygen tension, and probably elevation of the carbon dioxide tension, are essential for growth and migration on a free surface.

4. Consecutive transfers from slant to slant were effected, but separation of *E. histolytica* from bacteria was not achieved by this method.

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AN EXCEPTIONAL CASE OF OXYURIASIS OF THE INTESTINAL WALL

J. Bijlmer

From the Institute of Tropical Hygiene, Amsterdam

This case relates to a man, 46 years old, who during the last six months was suffering from diarrhoea. He was in a camp and remained there, his complaints not being considered serious. Three weeks before his admission to the hospital, his diarrhoea grew so violent, that he had to stop working and was sent home. At that time the patient was already in a precarious condition, exhausted, dull and exsiccated, and he had watery stools with blood, 10–20 times a day. Apart from a little pain at palpation of the colon, physical examination showed nothing particular. Blood pressure 110/80 mm Hg; Haemoglobin 115%; erythrocytes 6,240,000 per mm^3; slight leucocytosis 15,500 per mm^3; blood sedimentation 2 mm after one hour, 6 mm after two hours (Westergren). Serological and bacteriological reactions to typhoid, paratyphoid A and B and dysentery were negative. In spite of the abundant supply of fluids, administered subcutaneously as well as intravenously, the man succumbed the fourth day after arrival in the infirmary with symptoms of increasing uraemia. The last two days of his life the patient was incontinent, evacuating watery stools at every change of attitude.

Post mortem: The mucosa of rectum, colon and cecum showed a thin greenish coating on the swollen folds and more or less transverse red-violet spots up to $1\frac{1}{2}$ cm wide, with whimsically formed yellow ulcers. The ulcers were often situated near the mesenteric insertion and were frequently arranged in groups or rows. The ulcers contained thin feculent matter with bloodclots. The appendix showed nothing particular. About midway in the ileum there were two ulcers of 1.3 by 0.7 cm and somewhat lower down four little ones, all with a red-violet bottom.

Histological examination revealed a gangrenous ulceration in rectum, colon and ileum with numerous worms penetrating as far as submucosa and subserosa.

The pathologist's (Dr. M. Straub, Rotterdam) diagnosis said: "acute ulcerative exacerbation of a chronic entero-colitis throughout rectum and colon, and some ulcers in the ileum, due to oxyuriasis."

On account of the finding of nematodes, samples of the colon, rectum, liver, spleen and kidney, fixed in formalin were sent to the Institute for Tropical Hygiene in Amsterdam.

Small pieces of the bowels were teased under a binocular and we succeeded in extirpating some hundreds of worms, many of them undamaged. Pieces of the enteric wall with a square area of $\frac{1}{2}$–1 cm^2 were divided lengthwise so that one part consisted of mucosa, submucosa and muscularis circularis and the other part of muscularis longitudinalis, transmuscular fat and serosa as far as the bowels were covered with it. In this way it became clear which worms were inside the tunica muscularis and which were outside. Eight pieces of the colon and eleven pieces of the rectum were examined, from macroscopically sound parts as well as from ulcers. In all parts save one we discovered worms in varying quantities. The free worms

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were mounted in gelatine-glycerine, the edge of the cover-slips being sealed with cedarwood oil. On closer examination the worms proved to be *Enterobius vermicularis;* the females characterized by the long sharp tail (Figs. 2 and 3), the males by the spiculum (Figs. 1 and 11). The papillae on the hind parts of the males were difficult to see; there seemed to be six pairs (Hsü, 1933). Usually the alae cervicales were clearly visible, but not so broad and fine as in fresh adults.

Sometimes the worms were evenly spread throughout the intestinal wall; in other parts large accumulations of the parasites occurred. The worms in the transmuscular tissue were situated in more or less open spaces and so most undamaged specimens came from that part. When the nematodes were present transmuscularly they also appeared in the submucosa, but sometimes in lesser number. On the other hand in some places great numbers of parasites have been found in the submucosa without a single one being detected transmuscularly. From time to time we came across transmuscular clusters of worms enclosed in a rather solid sheath (Fig. 4). At first we thought it comparatively easy to detach these sheaths from the surrounding tissue; on closer inspection, however, all appeared to be damaged, anyway they were open and the worms were protruding from both ends. So, one could not tell whether these clusters of worms were confined in a hollow surrounded by a sheath on all sides or in a tunnel-like space. In these sheaths the nematodes were found either coiled or stretched in a bundle. Stained with carmine, the wall of these cavities showed nuclei, which suggested that they were a product of the host's tissue. In eight pieces of the colon with a total square area of 4 cm^2 there were more than eighty worms averaging about 20 per cm^2; 20 of them, that is 5 per cm^2, were lying in the subserosa and the remaining 60 in the submucosa. The proportion of males to females was 5: 1. From eleven pieces of the rectum with a total square area of 11 cm^2 we got more than 650 worms, that is an average of 60 per cm^2; about 200 were situated transmuscularly. The proportion of males to females here was 6: 1. Calculating from these figures, the total number of worms in the intestinal wall was far over 10,000 individuals, in the rectum as well as in the colon. The number of parasites in the lumen of the bowel must have been enormous, as it is not likely that all worms penetrated into the wall; moreover we found a large majority of males whereas the total number of both sexes is presumably equal. Curiously enough the patient's complaints did not suggest oxyuriasis at all.

All worms were very small, in particular the females. The average length of 172 males was 1550 μ and of 26 males 1677 μ. Not one female containing eggs has been found; some of them showed a scanty development of the genitals, but by far most of the females had not even an indication of those. On the other hand all males possessed full-grown genitals, in sections of which spermatogenesis could often clearly be demonstrated.

Another striking feature was that in a large number of females the intestine from bulbus to anus was crammed with strongly refractive granules (Fig. 3); this was found only once in a male. Others had larger and less refractive particles in their intestines (Figs. 1 and 2). Apparently the females could be divided into two classes. one with a relatively short esophagus and bulbus (Fig. 3) and the other with these organs relatively long (Fig. 2).

On Table 1 the data of all measured females are given. Large as well as small specimens have a long esophagus, but with the former it occurs more often. None

of the females with genitals had strongly refractive granules in the intestine and all minus signs in the fourth column correspond to a relatively long esophagus.

Perhaps we are dealing with two different stages of female larvae, the males presenting one larval stage only.

Sections taken from the same spots as the teased samples confirmed that very great numbers of worms were present in the wall of the intestine, most of them lying in the submucosa. The mucosa had been damaged at numerous spots and nearly each section contained one or more ulcers, the larger ones with deep undermined

TABLE I.—*Females*

Body-length in μ	Length of esophagus	Length of bulbus	Refractive granules	Genitals
	In % of body-length			
1200	12.3	5.1	+	–
1240	25.4*	7.3*	+	–
1320	14.2	5.5	+	–
1380	10.9	4.3	+	–
1380	18.6	6.5	+	–
1450	13.8	5.6	+	–
1480	11.6	4.9	+	–
1500	24.3	7.6	+	–
1570	22.3	7.4	–	+
1570	11.9	4.4	+	–
1580	11.8	4.4	+	–
1600	23.8	6.8	+	–
1600	12.3	4.8	+	–
1620	21.9	6.9	+	–
1690	11.0	4.1	+	–
1700	20.8	6.9	–	–
1750	24.2	6.4	+	–
1775	20.5	6.6	+	–
1800	21.1	6.0	–	+
1810	29.8	7.1	–	–
1920	20.1	6.5	–	+
1980	18.7	5.0	–	–
2000	21.8	6.2	–	+
2070	19.3	5.7	–	–
2275	21.2	6.1	–	–
2325	16.8	5.7	–	+

* Long ones underlined.

edges (Fig. 5). Therefore we looked in these sections for amebae, but without success. The ulcers were filled with a necrotic mass in which usually there were some worms. The worms were situated singly or in groups, in the submucosa as well as transmuscularly. In the submucosa the worms were concentrated around the ulcers and accordingly they were often surrounded by many leucocytes; sometimes the worms were lying in an abscess (Fig. 5). But very often the tissue did not seem to react at all to the invasion. When the worms were lying in groups they were usually enclosed in something like a sheath (Fig. 6) in accordance with the findings in the teased-out tissues. These sheaths were not always intact. Sometimes an inner layer without nuclei, perhaps produced by the parasites, could be distinguished from an outer layer of connective tissue. Other sheaths consisted of only one of

FIG. 1. *Enterobius* ♂ (59×).
FIG. 2. *Enterobius* ♀ with genitals (33×).
FIG. 3. *Enterobius* ♀ without genitals; strongly refractive granules in the intestine (54×).
FIG. 4. Twisted *Enterobius* in a sheath (47×).
FIG. 5. Section of an ulcus with undermined edge. Under the bottom of the ulcus two small abscesses, the left one containing an *Enterobius* (29×).
FIG. 6. Section of a bundle of *Enterobius* surrounded by a sheath; some are lying outside (59×).

these layers. Inside the inner layer a necrotic mass of variable dimensions was often visible. Around the incomplete sheaths there was no inflammation. Transmuscularly the absence of inflammation was still more conspicuous. One gets the impression that the worms themselves do not produce inflammation of the host's tissues, but that they may carry along some bacteria, sometimes giving rise to an abscess. Some worms, with or without a membrane were situated in a space of the same configuration, but much larger than the worm. The space between the cavity and the worm was too large to be a result of fixation. Moreover in the latter case all parasites would have shown this phenomenon. As endothelial cells in the wall have been found a few times only, it is not likely that all these cavities are lymph vessels. Other investigators also (Troisier et Deschiens, 1930) have described this phenomenon and Brumpt (1936, p. 875), believed he could recognize these cavities even when devoid of worms; he called them "cavités vermineuses."

Often the nematodes were lying together in great numbers in extensive gaps in the tissue (Fig. 7). In one of our sections it seems as if there is a connection between such a gap in the submucosa and one in the transmuscular layer (Fig. 9). In this case the breach through the muscles is situated where they are penetrated by large blood-vessels, i.e., where the tunica muscularis is interrupted and weakened. In other sections, too, the worms have been found situated transmuscularly near places where large vessels were crossing the muscular layer. In the tunica muscularis itself only two worms have been traced. Once a transverse section has been found of a worm surrounded by a necrotic mass, the worm trying to penetrate the muscular layer (Fig. 8). The last muscle fibers that were still barring its way had already been pushed back. Curiously enough, neither inflammatory cells, nor endothelial cells could be discovered, which gives rise to the supposition that the worm forces its way by causing an aseptic necrosis. On the other hand the presence of lymph was observed, which suggests that the worm was trying to pass the tunica muscularis by a lymph space. That the worms can penetrate into lymph vessels is certain, as we found them even in rather large blood-vessels (Fig. 10). These last findings induced us to examine the liver whither the parasites might have been carried along with the blood-current, but no parasites were found in sections and in teased tissue.

Serial sections from several pieces of the intestinal wall showed that nearly all the worms in the submucosa lay in spaces connected with the intestinal lumen. Consideration of all these facts suggests that the nematodes entered the tunica submucosa by way of little ulcera and then penetrated the tunica muscularis at a weak spot.

As to the etiology of the findings three possibilities present themselves: (1) *Enterobius* was the cause of the gangrenous ulceration, (2) *Enterobius* penetrated through a pre-existing gangrenous ulceration during life, (3) *Enterobius* invaded the ulcers after death. In all probability the ulcers pre-existed and the helminthic invasion was a secondary phenomenon.

Nevertheless it is quite possible that the worms sometimes imported bacteria into the tissues.

Our findings closely agreee with those of Cecil and Bulkley (1912). The ulcers with undermined edges indicate that *Enterobius* exerts at least a typical influence on the ulceration. The tunnels strengthen the conception that the invasion occurred during the life of the patient.

It remains remarkable that the most extensive invasion has been observed in the

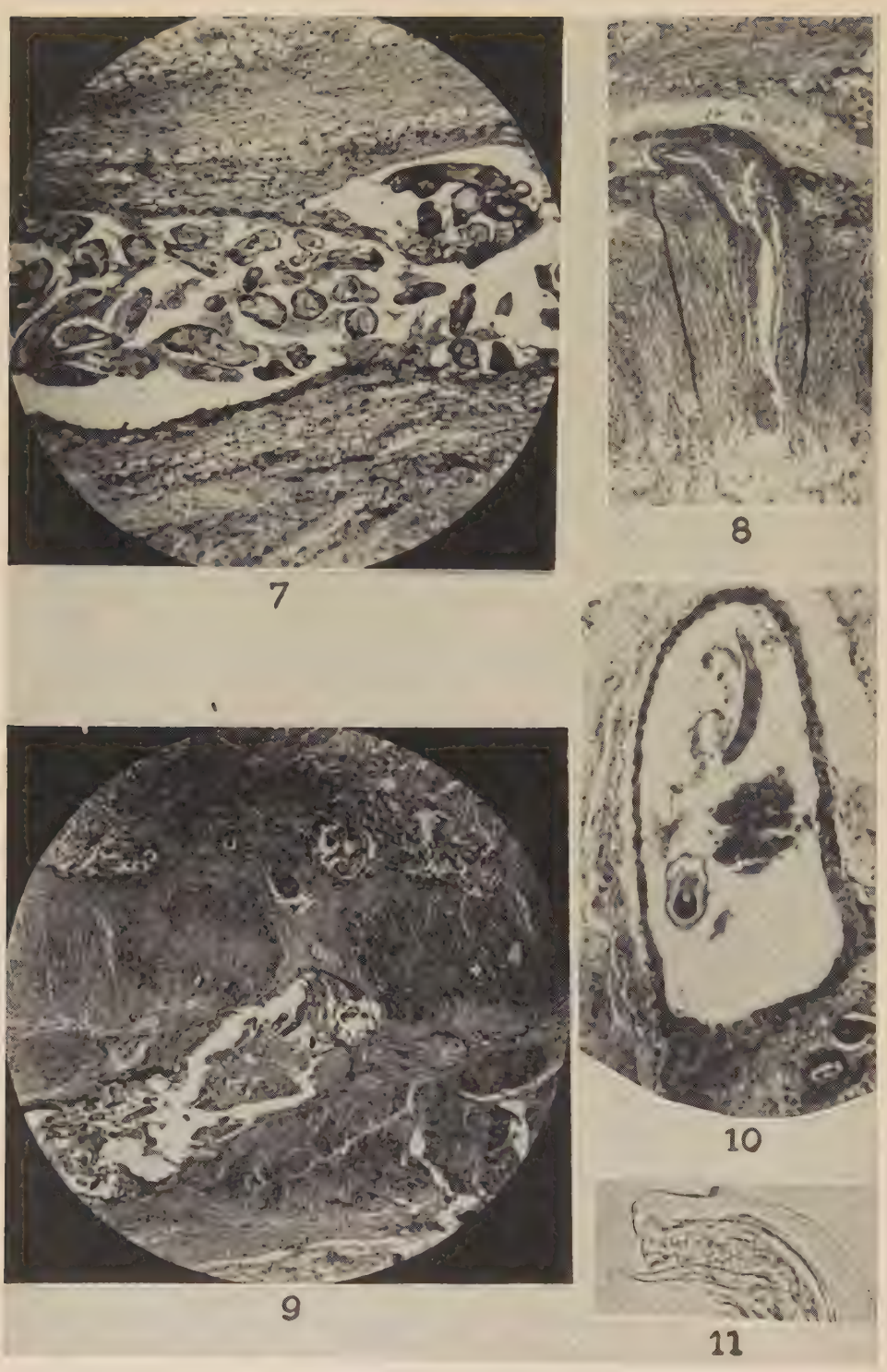

Fig. 7. Section of a gap in the transmuscular tissue with numerous *Enterobius* (59×).

Fig. 8. Same gap as in fig. 7 where it is breaking through the muscular layer into an ulcus. On the same spot two large blood-vessels are to be seen (17×).

Fig. 9. Section of an *Enterobius* surrounded by lymph and necrotic mass pushing through the tunica muscularis (28×).

Fig. 10. *Enterobius* in a vein (64×).

Fig. 11. Spiculum (338×).

wall of the rectum, *Enterobius* normally inhabiting the cecum and adjacent parts. Usually only pregnant females find their way to the rectum.

In literature, a case with an invasion of many thousands of nematodes in the intestinal wall is unknown. Most papers refer to the appendix, only few authors mentioned periproctitic abscesses with *Enterobius* inside, or, invasions of the intestinal wall outside the appendix (Vuillemin, 1902; Ruffer, 1901; Nathan, 1928). Rheindorf (1920) only mentioned in his monograph on *Enterobius* that he had found, among others, very young worms in the intestinal wall.

In literature, opinions are divided as to whether *Enterobius* is a cause, perhaps a frequent cause of appendicitis or not (Rheindorf, 1920; Cecil and Bulkley, 1912; Brumpt, 1936; Wood, 1918; Läwen and Reinhard, 1920; Schwarz and Straub, 1940; Unterberger, 1908; versus Aschoff, 1912 and 1913; Sagredo, 1914; Matsuoka, 1916; Fischer, 1923; Brauch, 1923; and Gordon, 1933).

SUMMARY

We found in our case an invasion of tens of thousands of *Enterobius* in the intestinal wall, especially in that of the rectum, but also in that of the colon and the ileum. There were five to six times as many males as females. All worms were still in a larval stage, but the males were one or two larval stages ahead of the females. Most striking were the many ulcers with undermined edges in the intestinal mucosa from where the invasion had obviously spread, and furthermore the almost complete absence of inflammation around the penetrated parasites. Evidently the reaction of the tissue consisted in enclosing the worms. Curious also was the low blood sedimentation.

It remains unsettled which was pre-existent, the ulceration or the invasion of the worms. The author himself leans to the first interpretation.

I wish to express my sincere thanks to Prof. Dr. S. L. Brug (of the Institute) under whose skilful direction this investigation has been accomplished and to Dr. M. Straub (Rotterdam) who drew our attention to this case and was so kind as to send us the material.

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FURTHER STUDIES ON DISTRIBUTION OF SCHISTOSOMIASIS JAPONICA IN SZECHWAN PROVINCE, CHINA

SHAO CHOU KUO

National Central Univ. Med. College, Chengtu, China

Kuo, Yui and Chang (1945) reported on a field survey of schistosomiasis japonica in Szechwan. Since the publication is not generally known in the United States, it is pertinent to mention briefly the results of the previous work, in addition to further studies.

Faust and Meleney (1924) reported the first case of schistosomiasis japonica in Jen Shou district, Szechwan; but until recently only few cases were reported in other districts. There are now two districts definitely known to have autochthonous cases of schistosomiasis, with nine others considered to be possible endemic areas. Kuo et al visited only five of the districts and in four of them Kuo found snails which looked like a species of *Katayama* (see Bartsch, 1936), and which he believed to be possible intermediate hosts of *Schistosoma japonicum,* although he failed to demonstrate the presence of *S. japonicum* cercariae on examination of more than 2,000 snails.

At a later date, Y. T. Yao made an intensive study of schistosomiasis in Pengshien, one of the districts where Kuo found the *Katayama* snails. After spending nearly a month there, he found (personal communication) about 20 fresh cases of schistosomiasis in school children, diagnosed by finding the ova in the stools. He also examined more than 4,000 specimens of the snail, and found one harboring cercariae of *Schistosoma japonicum,* thus proving that the snail could really be the intermediate host.

On coming to the United States, Kuo brought the snail to Dr. Paul Bartsch of the Smithsonian Institution for identification, and it was found to be identical with the specimens described by Bartsch (1946) as *Schistosomophora slateri.* These specimens were sent from Chengtu by Slater who obtained them from Yao. Lt. Tucker Abbott of Harvard University, however, held the opinion that *S. slateri* was merely a form or subspecies of *Oncomelania nosophora.*

Besides the study of human schistosomiasis, Kuo also directed his attention to bovine schistosomiasis. Hsiung (1943) in Chengtu examined 50 cattle mesenteries and found 26% of them infected with *Schistosoma japonicum,* while 36% showed infection with *Ornithobilharzia sp.* Kuo, working on 41 cattle mesenteries, confirmed Hsiung's report and found infections of 17.07% with *Schistosoma japonicum* and 41.46% with *Ornithobilharzia sp.*

In Hsiung's work, the cattle were said to have come from various districts. In Kuo's cases, they were said to have come from the north and south routes, Ying Shan and Ta Ching Shan districts, both some distance from Chengtu, indicating a wide range of distribution of schistosomiasis.

Thus it can safely be predicted that the distribution of schistosomiasis in Szechwan Province must be much wider than the areas at present known, although the incidences of infection may not be so great as in the provinces further downstream, along the Yangtse River.

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The presence of *Ornithobilharzia sp.* in the cattle is also of some interest. About 40% of the cattle slaughtered in Chengtu suffered from such infection. The small size of the blood flukes and the presence in some female specimens of eggs not quite similar to *Ornithobilharzia turkestanicum* led Kuo to suspect the presence of a new species. But careful study of the specimens while in New York led him to believe that they are *Ornithobilharzia turkestanicum* (= *Schistosoma turkestanicum* Skrjabin, 1913).

For the study of *Ornithobilharzia* the writer is indebted to Dr. H. W. Stunkard for his kindly advice and guidance, and to Dr. Dominico L. DeGiusti for his help and advice in the preparation of the specimens.

SUMMARY

1. Cases of schistosomiasis japonica are now known from eleven districts in Szechwan, a province formerly thought to have few cases of schistosomiasis.

2. The presence of *Schistosoma japonicum* in the mesenteries of cattle coming from different districts indicates a wider range of distribution.

3. A new subspecies of snail has been found to be capable of acting as intermediate host for *S. japonicum.*

4. In addition to *S. japonicum, Ornithobilharzia turkestanicum* is common in the mesenteries of cattle of Szechwan.

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STUDIES IN TRYPANOSOMIASIS.[1] I. THE PLASMA PROTEINS AND SEDIMENTATION RATES OF ERYTHROCYTES OF RATS INFECTED WITH PATHOGENIC TRYPANOSOMES

OKECHUKWU IKEJIANI[2]

Department of Pathology and Bacteriology, University of Toronto

An accelerated sedimentation rate of the red blood cells has been observed in various pathological conditions. Newham and Martin (1928) have reviewed the literature on the various pathological conditions in which the sedimentation rate of the red blood cells is accelerated. It can be stated generally that increased sedimentation rate of the red blood cells has been observed in acute infections of all types, in anemia, and in conditions associated with extensive destruction of tissue. For these reasons, therefore, increased sedimentation rate, as a rule, is not diagnostic of any disease, although it can be taken as a corroborative evidence of certain types of disease.

The factors responsible for the increased sedimentation rate of the red blood cells and the mechanism of the process involved are as yet not fully understood. Most investigators, however, have reported varying degrees of correlation between increased rate of red blood cell sedimentation and increased globulin and fibrinogen content of the plasma. In particular, Fahreus (1929), in his classical investigations of the phenomenon of rapid sedimentation, has surveyed the literature on the subject of the protein proportions of the blood in rapidly sedimenting cases and has stated that the survey revealed that fibrinogen or serum globulin or both were increased in those conditions when it was known or suspected that the settling velocity of the red blood cells was increased.

In the course of studies in experimental trypanosomiasis it became necessary to study the levels of plasma proteins, since the results of investigations with formol-gel, mercuric chloride, aldehyde and several other tests proposed for the diagnosis of trypanosomiasis, indicated clearly that there was a marked disturbance in the serum protein fractions during the course of infection. Furthermore, the sedimentation rate during the course of infection with pathogenic trypanosomes was studied in an attempt to determine whether a change in the serum protein was responsible for the increased sedimentation rate observed in these infections.

Sicé, Boisseau, Provost and Deniel (1931) and Sicé and Bonnet (1936) studying the relationship of the serum albumin to serum globulin observed that the ratio $\frac{\text{serum albumin}}{\text{globulin}}$ was always less in human cases of trypanosomiasis than in normal persons. The lowering of the quotient was due to the increased serum globulin. Newham and Martin (1928) found very little change in the levels of plasma proteins in a case of human trypanosomiasis in which treatment with tryparsamide had

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[2] The author wishes to express his appreciation to Dr. Lawrence H. Mautner of the Department of Pathology and Bacteriology, University of Toronto, for his helpful suggestions and criticisms.

been started. French (1938) reported decreased albumin in animals infected with *T. congolense* and *T. brucei.*

On the sedimentation rate, the earliest work was that of Amalfitano (1935) who studied the sedimentation rate in sheep infected with *T. gambiense* and *T. brucei.* He reported acceleration of sedimentation of the red blood cells in all cases and assumed that the presence in the plasma of products of metabolism and lysis of the parasites played an important rôle in the accelerated sedimentation rate observed.

METHOD

Three groups of rats were used in these experiments. The first group served as controls and the others were infected with *T. brucei* and *T. equiperdum,* respectively. To produce a uniform result, female rats were used in all the experiments since preliminary investigation showed a great variation in the rate of sedimentation of red blood cells between the male and female rats.

Trypanosome and red blood cell counts were determined according to the method described by Zwemer and Culbertson (1939). Serum proteins were determined by the method of Campbell and Hanna (1937) which depends on the precipitation of the globulins by 20 per cent sodium sulfite. The total nitrogen of the resulting filtrate and of diluted serum was determined by the micro-Kjeldahl procedure. After correction for non-protein nitrogen, values were converted to protein by being multiplied by 6.25. Globulin concentration was calculated as the difference between the total protein and albumin concentrations.

The sedimentation rate was determined by the use of a modified Westergren technic (1920). Oxalated blood was drawn into a sedimentation pipette (1.5 mm inside diameter) to a height of 200 mm. The end of the pipette was immersed in mercury to prevent the contents from running out, and the pipette was supported in a perpendicular position at room temperature. At intervals of 1, 2, 6, and 24 hours, the clear supernatant fluid which resulted from settling of the red blood cells was measured in mm.

RESULTS

The Sedimentation Rate of Erythrocytes and Serum Proteins in Normal, T. brucei *and* T. equiperdum *Infected Rats*

The results of the experiments with normal rats are shown in Table 1, while those of *T. brucei* and *T. equiperdum* infected rats are shown in Tables 2 and 3, respectively. All rats infected with *T. brucei* or *T. equiperdum* showed an ac-

TABLE 1.—*Plasma proteins and erythrocytic sedimentation rate of normal rats*

Rat No.	Total protein	Albumin	Globulin	Albumin / Globulin	Erythrocyte sedimentation rate in mm at designated hours			
					1	2	6	24
	gm%	*gm%*	*gm%*					
1	6.3	3.9	2.4	1.7	1.2	2.8	5.3	9.8
2	5.8	3.4	2.4	1.4	1.5	3.0	6.5	10.0
3	5.9	4.0	1.8	2.1	1.7	2.1	4.7	8.0
4	5.7	3.9	1.8	2.1	1.2	3.4	7.5	11.5
5	5.8	4.0	1.8	2.1	1.6	3.9	5.7	8.2
6	5.9	3.8	2.1	1.7	1.4	2.8	6.0	8.5
7	6.4	4.0	2.4	1.6	1.8	2.5	6.2	9.2
8	6.2	4.1	2.1	1.8	1.4	2.8	5.8	8.1
9	5.7	3.5	2.2	1.6	1.6	3.4	6.9	10.5
10	5.8	3.5	2.3	1.7	1.5	3.3	6.3	9.6
Ave.	5.95	3.81	2.13	1.8	1.5	3.0	6.1	9.34

TABLE 2.—*The erythrocyte sedimentation rate, plasma proteins, trypanosome and red blood cell counts of rats infected with* Trypanosoma brucei

Rat No.	No. of trypanosomes per cmm	No. of RBC per cmm	Total protein	Albumin	Globulin	Albumin / Globulin	Erythrocyte sedimentation rate at designated hours			
							1	2	6	24
			gm%	*gm%*	*gm%*					
1	900,000	3,750,000	5.8	2.8	3.0	0.9	4.0	8.0	15.4	37.0
2	780,000	5,200,000	..	..	..	..	5.0	10.2	16.8	50.2
3	670,000	4,000,000	6.0	2.9	3.1	0.9	4.2	8.7	20.5	82.0
4	950,000	3,200,000	5.4	2.4	3.0	0.8	4.8	12.5	48.1	85.1
5	1,017,000	3,000,000	..	..	..	..	4.5	8.9	22.5	65.0
6	900,000	4,100,000	6.5	2.9	3.6	0.9	3.7	6.5	18.8	48.3
7	1,012,000	2,450,000	..	..	..	..	3.9	5.7	14.4	48.9
8	675,000	5,200,000	6.3	3.2	3.1	1.0	4.7	7.5	13.7	63.0
9	730,000	4,100,000	..	..	..	..	4.9	13.5	24.5	64.0
10	980,000	3,500,000	5.7	3.3	2.4	1.4	5.5	13.6	30.2	84.5
Ave.	861,400	3,850,000	5.95	2.91	3.03	1.0	4.52	9.51	22.5	62.8

celerated sedimentation velocity, decreased albumin and increased globulin serum proteins with an alteration in the albumin-globulin ratio.

If graphs are plotted with time in X axis and the sedimentation rate in mm in Y axis, curves would be obtained from the average of figures in Tables 1, 2 and 3. It can easily be seen that the sedimentation curves from normal rats differed from those of infected rats. With normal rats, the sedimentation curve flattened after 24 hours and was almost parallel with the asymptote while the sedimentation curve for infected rats was accelerated from the start and was maintained during the 24 hours. This can be explained only by assuming that during 24 hours the rate of sedimentation was constant in infected rats, whereas in normal rats this was not the case.

The sedimentation rate was quite independent of the number of trypanosomes present in the circulating blood. One of the greatest sedimentation rates (rat 6, Table 3) was obtained in a rat in which the trypanosome counts was about the lowest in the group. The relationship between sedimentation rate and the number of trypanosomes was therefore not clear cut.

The degree of anemia actually coincided with the greatest rate of sedimentation, although there seems to be no absolute relationship here. This seems however to suggest that the red blood cells play an important rôle in the increased sedimentation rate observed in pathogenic trypanosome infections.

Attempt to Determine the Factors Responsible for the Increased Sedimentation Rate in Pathogenic Trypanosome Infections

Samples of blood of rats infected with *T. brucei* were centrifuged at 1000 revolu-

TABLE 3.—*The erythrocyte sedimentation rate, plasma proteins, trypanosome and red blood cell counts of rats infected with* Trypanosoma equiperdum

Rat No.	No. of trypanosomes per cmm	No. of RBC per cmm	Total protein	Albumin	Globulin	Albumin / Globulin	Erythrocyte sedimentation rate at designated hours			
							1	2	6	24
			gm%	*gm%*	*gm%*					
1	1,000,000	3,700,000	6.2	3.0	3.2	0.9	6.2	10.9	20.5	62.1
2	865,000	5,400,000	6.5	2.8	3.7	0.7	5.7	11.5	19.9	54.5
3	1,012,000	2,000,000	5.4	2.8	2.6	1.1	6.5	15.4	27.5	120.7
4	950,000	3,700,000	6.2	3.0	3.2	0.9	4.9	9.7	32.5	70.5
5	780,000	4,100,000	..	..	..	..	5.7	12.5	42.7	92.3
6	700,000	3,200,000	5.8	2.9	2.9	1.0	6.5	13.2	39.9	102.5
7	1,070,000	4,200,000	5.9	3.0	2.9	1.04	3.9	10.7	22.3	43.7
8	1,120,000	2,700,000	5.7	2.7	3.0	0.9	4.8	14.8	22.2	88.4
9	1,000,000	4,100,000	..	..	..	..	7.0	15.7	50.0	105.7
10	800,000	5,250,000	5.1	2.1	3.0	0.7	5.5	13.0	18.3	60.3
Ave.	929,700	3,835,000	5.85	2.8	3.06	0.9	5.7	12.74	29.6	80.07

tions per minute and the plasma pipetted off and stored in a refrigerator until used. The red blood cells were washed three times with physiological saline solution. This was also done with samples of pooled blood from normal rats. The plasma taken from *T. brucei* infected rats was added to the washed red blood cells of normal rats, and the plasma of normal rats was added to the washed red blood cells of *T. brucei* infected rats. Each sample was now shaken in order to mix the red blood cells. Erythrocytic sedimentation rate was observed.

The results of the experiments are shown in Table 4. The results show that the addition of the plasma of the rapidly sedimenting *T. brucei* infected rats to the corpuscles of normal sedimenting ones produced little or no significant results in the sedimentation velocity. On the other hand, when the red blood cells of the rapidly sedimenting infected rats were added to the plasma of the normal sedimenting ones, a distinctly accelerated sedimentation rate was observed.

These results would seem to indicate that whatever factor or factors are responsible for an abnormal rate of sedimentation, appear to reside in some of the properties

TABLE 4.—*Comparative sedimentation rate of normal and* Trypanosoma brucei *infected rats with the sedimentation rate of rapidly sedimenting plasma of* T. brucei *infected rats plus the corpuscles of normal rats and the rapidly sedimenting corpuscles of* T. brucei *infected rats plus the plasma of normal rats*

Group of rats	No. of rats in each group used	Description	Sedimentation rate in mm at designated hours			
			1	2	6	24
1	10	Normal rats (from Table 1)	1.5	3.0	6.1	9.34
2	10	*T. brucei* infected rats (from Table 2)	4.52	9.51	22.5	62.8
3	5	Pooled corpuscles of normal rats plus the plasma of *T. brucei* infected rats above	1.6	3.5	7.0	12.8
4	5	Pooled corpuscles of *T. brucei* infected rats above plus the plasma of normal rats	4.0	9.2	21.5	55.7

of the red blood cells rather than in any particular change in the plasma and its constituents.

It is of interest, however, to note that when the plasma of the rapidly sedimenting blood was removed and replaced by physiological saline solution, the rate of the sedimentation was found to fall within the normal range. It must be emphasized here, however, that one is introducing a very marked change in the conditions. Not only is the specific gravity and viscosity of the physiological saline solution very different from that of the plasma, but none of the chemical constituents of the plasma other than sodium and chloride are present.

DISCUSSION

The change in serum proteins observed in these experiments is interpreted as due to the degeneration of the kidney observed in pathogenic trypanosome infections by Andrews, Johnson and Dormal (1930), and unpublished studies by Mautner and Ikejiani. The damage done to the kidney during pathogenic trypanosome infection must have injured the selective excretory mechanism by which the kidney aids in maintaining a constant blood plasma protein level.

When the loss of protein, chiefly albumin, results from infections or from lipoid nephrosis the serum albumin-globulin ratio falls. In the plasma, the concentration of the globulin fraction may actually exceed that of the albumin fraction, i.e.,

the normal ratio is inverted. This is shown by the fact that albumin was detected in the urine specimens of rats infected with pathogenic trypanosomes.

The change in the serum proteins does not seem to be responsible for the accelerated sedimentation rate observed in these experiments since it has been shown that the factor or factors responsible for the increased sedimentation rate seem to reside in the red blood cells rather than in the plasma.

SUMMARY

There was an increase of globulin, a decrease of albumin and altered albumin-globulin ratio of the serum proteins of rats infected with *T. brucei* and *T. equiperdum*. It is suggested that kidney damage is responsible for the altered serum proteins.

The sedimentation of the blood of rats infected with pathogenic trypanosomes increased; however, it is suggested that the altered plasma protein is not the cause of increased sedimentation rate in pathogenic trypanosome infections.

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STUDIES IN TRYPANOSOMIASIS.[1] II. THE SERUM POTASSIUM LEVELS OF RATS DURING INFECTION WITH *TRYPANOSOMA LEWISI, TRYPANOSOMA BRUCEI,* AND *TRYPANOSOMA EQUIPERDUM*

OKECHUKWU IKEJIANI[2]

Department of Pathology and Bacteriology, University of Toronto

Practically all investigations in blood chemistry alterations resulting from trypanosome infections in animals and man have been limited to the studies of the blood sugar and red blood cell counts. A few data are available concerning the carbon dioxide combining power, hydrogen ion concentration and lactic acid of the plasma. Literature regarding the alterations of inorganic elements of the blood during trypanosome infections is limited. Very little work has been done in this field and it has dealt chiefly with the effects of the various elements on trypanosomes *in vitro* or with their influence on the course of infection.

Kliger, Geiger and Camaroff (1929) showed that the removal of any important cation from the diet of rats infected with *T. evansi* reduced the survival time of the rats. Simons (1918) showed that calcium was beneficial for the survival of trypanosomes *in vitro,* while Henderson (1931) showed that pica could be prevented in cattle infected with trypanosomes by feeding the animals with calcium and sodium. They assumed that both calcium and sodium prevented the acidosis which developed in these animals during the course of infection. Sicé (1930), studying the nervous cases of human trypanosomiasis, found that the spinal fluid is modified not only by the leucocyte reaction and increase in the albumin but by a diminution in the quantity of chlorides and sugar of the cerebrospinal fluid.

The only references known to the author dealing with the inorganic blood contents during infection with trypanosomes are the investigations of Linton (1930) who found no variation in the blood chlorides of rats infected with *T. equiperdum,* and Zwemer and Culbertson (1939) who found an increase in the levels of potassium of the serum of rats infected with *T. equiperdum.* The last-named authors have considered the increase in the plasma potassium as perhaps due to the differential permeability of the red blood cells as a result of parasitic infection.

The experiments reported here show that there is an increase in serum potassium and a decrease in red cell numbers during infection with pathogenic trypanosomes while no significant changes were observed in infection with non-pathogenic trypanosomes.

METHOD

The methods of infecting the rats and counting the trypanosomes have been described by Zwemer and Culbertson (1939). Potassium determination was made by the method of Truszkowski and Zwemer (1937).

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[2] The author wishes to express his appreciation to Dr. Lawrence H. Mautner of the Department of Pathology and Bacteriology, University of Toronto, for his helpful suggestions and criticisms.

Blood was obtained from the rats before and after infection by heart puncture under slight anaesthesia. A dry syringe was employed to prevent hemolysis of the red blood cells. The blood was allowed to clot in a long slant and the serum was poured off after it was squeezed out of the clot. Every effort was made to prevent hemolysis and any sample that became hemolyzed was discarded. For determination, 0.2-cc samples of serum were used and dilutions were made when increased potassium of the serum was observed. Difficulties in determination of potassium were encountered during the preliminary experiment, but after following instructions of Zwemer (personal communication) these handicaps were overcome. The handicap was due to insufficient washing and centrifuging of the very small amount of precipitate and failure to draw off completely the supernatant of the last washing. Potassium determinations were made on each rat before and after infection. All animals used were albino rats and no attempt was made to determine their age or weight. Trypanosome counts were also made at each time that the animals were bled for potassium determination. For erythrocyte counts, separate groups of rats were used, each group infected with *T. lewisi, T. brucei,* and *T. equiperdum,* respectively. Counts were made at intervals before and after infection. In these groups of rats no blood was taken for any other purposes.

RESULTS

Potassium Levels, Erythrocyte and Trypanosome Counts During Infection with Trypanosoma lewisi

The results of the experiments are shown graphically in Fig. 1. There was no significant change in the serum potassium levels of rats during the course of infection with *T. lewisi* (Fig. 1). The average potassium level for the normal rats was 21.4 mg per cent, while the potassium level during the height of infection was 23.8 mg per cent. The average potassium level when the trypanosomes had practically disappeared from the blood stream was 22.1 mg per cent.

One of the rats used for the erythrocyte and trypanosome counts died on the 30th day after infection. The cause of death was not determined, but it could not be attributed to any injury on the part of the parasites since the erythrocyte count was normal and the parasites had practically disappeared from the blood stream. One of the rats did not show any appreciable anemia. The relationship of the erythrocyte counts and the potassium levels as shown in Fig. 1 reveals that there was a diminished erythrocyte count on the 16th day after infection but soon this returned to normal.

Potassium Levels, Erythrocyte and Trypanosome Counts During the Course of Infection with T. brucei *and* T. equiperdum

The results of the experiments are shown graphically in Fig. 2. All the animals infected with *T. brucei* experienced a typical course of infection; the terminal trypanosome counts being between 910 thousands and 1,200 thousands per cmm of tail blood. The figure shows that rats infected with *T. brucei* suffered definite anemia. It is of interest to note that in all these rats the anemia observed was much more pronounced during the late stages of infection. This anemia could not be attributed to the successive bleedings from the tail for uninfected rats subjected to the same procedure did not vary very significantly in the red cell counts. Furthermore, those animals with *T. lewisi* infection which recovered, regained their normal cell counts although bleeding was still carried on.

The results of the experiments in rats infected with *T. equiperdum* are essentially similar to those obtained in infection with *T. brucei,* although the average serum potassium level seemed to be higher in *T. brucei* infection.

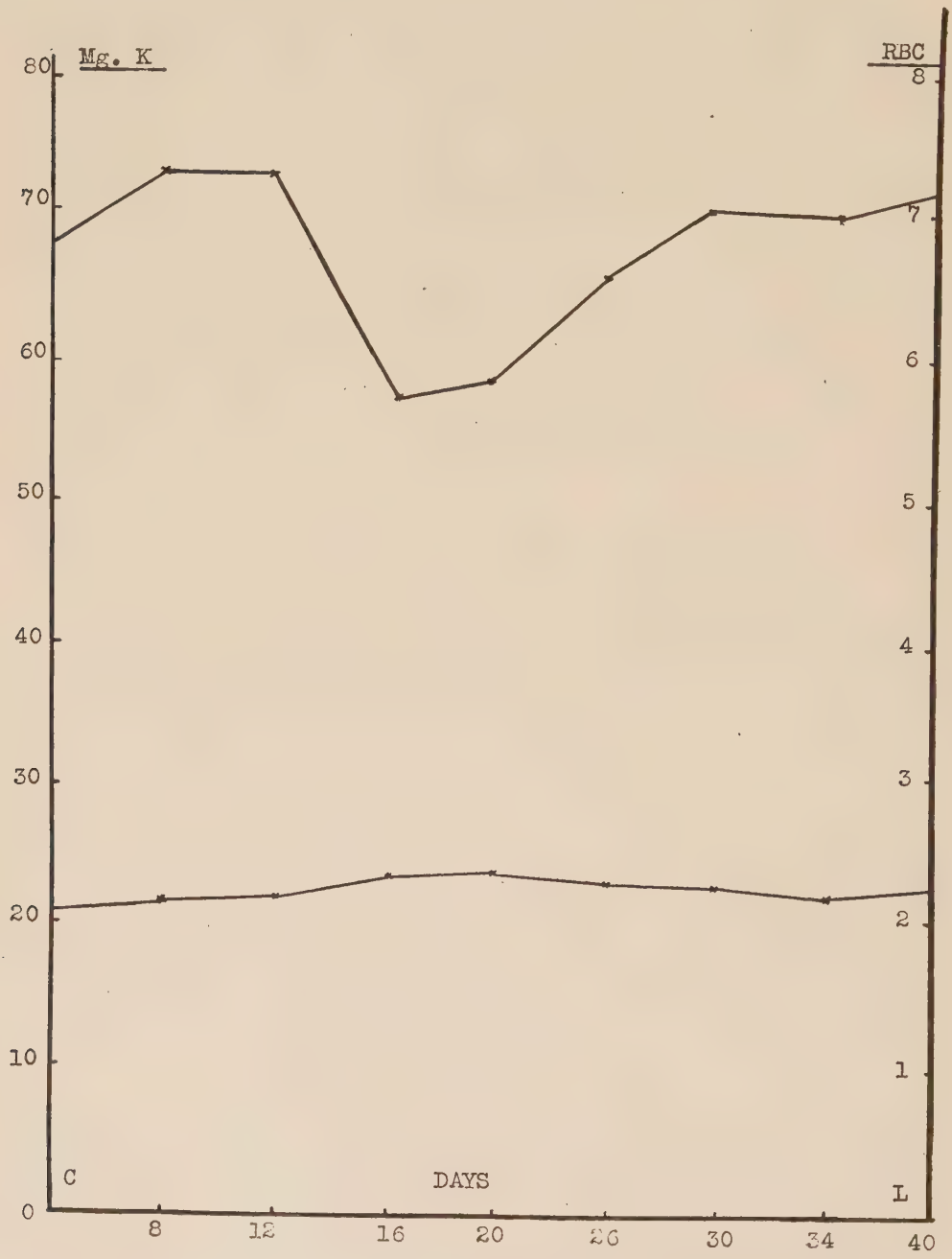

FIG. 1. Serum potassium and red blood cell changes during *Trypanosoma lewisi* infection in rats. (Serum potassium in mg per 100 cc, below; red blood cells in millions per cmm, above. On the abscissa, C refers to control samples, and L refers to the last samples taken when the trypanosomes had disappeared from the blood stream, 40 days after infection.)

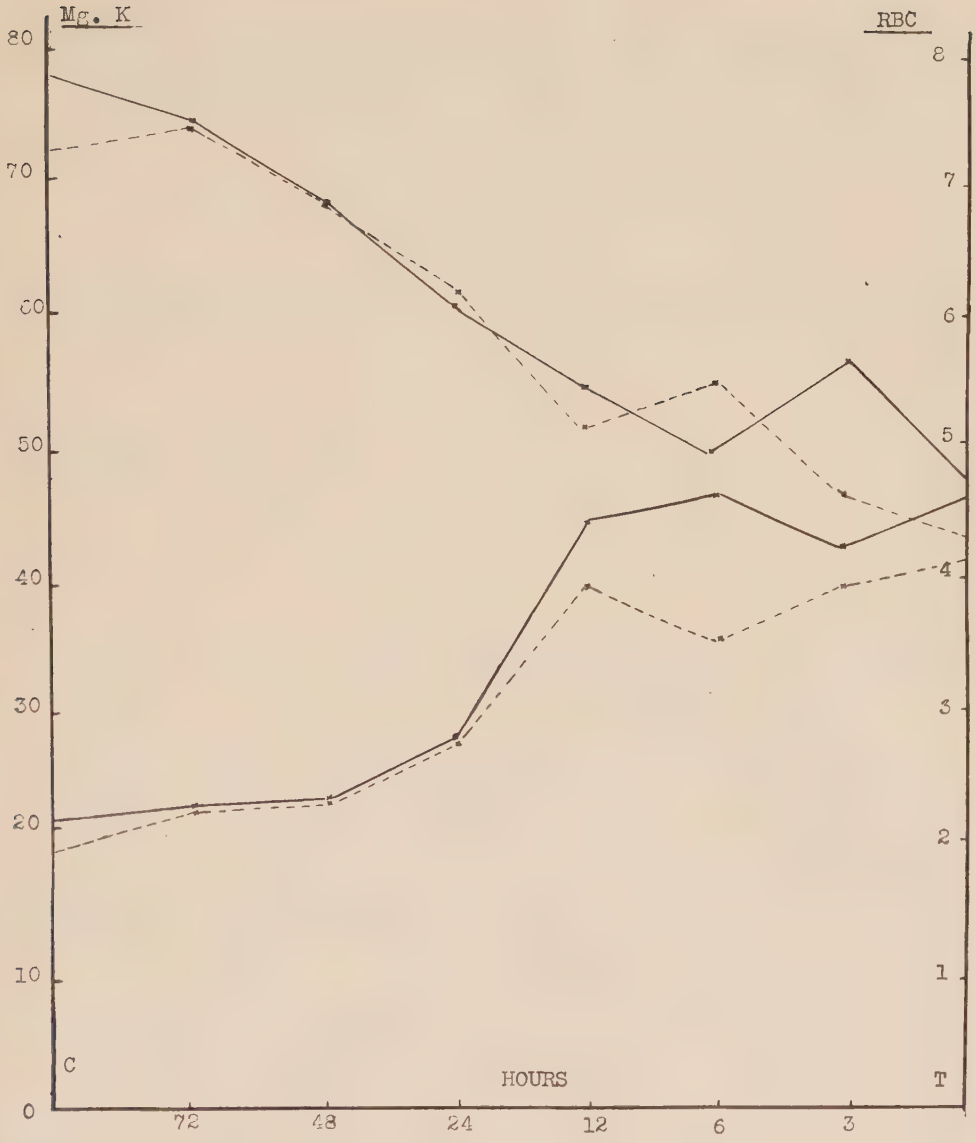

FIG. 2. Serum potassium and red blood cell changes during *Trypanosoma brucei* and *Trypanosoma equiperdum* infection of rats. (Serum potassium in mg per 100 cc, below; red blood cells in millions per cmm, above. On the abscissa, C refers to control samples, T refers to terminal sample, and the hour to the times before death when the blood samples were taken.) The lines in dashes represent *T. equiperdum* RBC and K, while the solid lines represent *T. brucei*.

DISCUSSION

The erythrocyte counts in infections with *T. lewisi* and *T. equiperdum,* but not in *T. brucei,* show a temporary increase in the peripheral circulation during the early stages of infection. This can best be interpreted as due perhaps to an early stimulation by the trypanosomes before the subsequent anemic activity is manifest. Thus, an early increase in the red blood cell count or red cell production is soon followed by red cell reduction as shown by progressive anemia. No explanation is offered for the absence of such a temporary increase in *T. brucei* infection.

There is quite a difference between the serum potassium levels of rats infected with *T. lewisi* on the one hand, and *T. brucei* and *T. equiperdum* on the other. The relationship between this increase in potassium levels and the erythrocyte counts as given in Fig. 2 suggests that the increased serum potassium might have come from the red blood cells since the figure shows quite clearly that an increase in potassium is accompanied by a decrease in the red cell count. This result confirms the work of Zwemer and Culbertson (1939). No such increase of relationship was observed in rats infected with *T. lewisi.*

A comparative study of the serum and erythrocyte potassium will clear the evidence, however, as to whether the increase in serum potassium was solely due to the decrement of the potassium of the red blood cells during pathogenic trypanosome infection.

SUMMARY

Rats infected with pathogenic trypanosomes (*T. brucei* and *T. equiperdum*) suffer anemia and increase in the serum potassium. This was not the case with rats infected with non-pathogenic trypanosomes (*T. lewisi*). There is some suggestion that the increased serum potassium during infection might have come from the red blood cells.

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STUDIES IN TRYPANOSOMIASIS.[1] III. THE PLASMA, WHOLE BLOOD AND ERYTHROCYTE POTASSIUM OF RATS DURING THE COURSE OF INFECTION WITH *TRYPANOSOMA BRUCEI* AND *TRYPANOSOMA EQUIPERDUM*[2]

OKECHUKWU IKEJIANI[3]

Department of Pathology and Bacteriology, University of Toronto

In order to ascertain whether the increased serum potassium observed during pathogenic trypanosome infections in rats as shown by the investigations of Zwemer and Culbertson (1939) and Ikejiani (1946), was due to the flow of substances from the red blood cells to the serum, experiments were planned to determine the potassium levels of the plasma, whole blood, and erythrocytes; from these relationships, one could judge whether the increase was the result of the decrement of the erythrocyte potassium.

METHOD

Albino rats were used throughout the experiments. Plasma, whole blood and calculated erythrocyte potassium determinations as well as trypanosome counts were made on the rats before and after infection until death.

For potassium determination, the method of Truszkowski and Zwemer (1937) was employed. At first, attempts were made to determine the red cell potassium obtained by centrifuging whole blood, washing the red cells twice with 0.85 per cent sodium chloride solution and bringing the red cell volume to original volume of the whole blood. The results obtained were not satisfactory because during the process of washing some potassium was lost from the red blood cells. Later, the following indirect method was employed: samples of blood were withdrawn by heart puncture from the rats with a dry syringe into a small test tube containing a little powdered sodium oxalate to prevent clotting. The contents of the tube were immediately centrifuged and the plasma was pipetted off the red cells and stored in the ice box until analyzed. It should be stated that prior to centrifugation, a sample of the whole blood was pipetted off for potassium analysis of the whole blood, 0.1-cc samples being employed. For plasma potassium, 0.2-cc samples were employed and the plasma was diluted as the potassium increased. For erythrocyte potassium, an indirect method of estimation was used. This consisted of making microhematocrit determinations for calculation of the erythrocyte potassium from the plasma and whole blood potassium. To calculate the erythrocyte potassium in milligrams per cent, the plasma potassium in milligram per cent was multiplied by the percentage plasma volume. The result was subtracted from whole blood potassium in milligram per cent and this was divided by the percentage red cell volume; that is, whole blood K mg% – (Plasma K mg% × % plasma volume) ÷ % red cell volume = erythrocyte K in mg%.

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[3] The author wishes to express his appreciation to Dr. Lawrence H. Mautner of the Department of Pathology and Bacteriology, University of Toronto, for his helpful suggestions and criticisms.

TABLE 1.—*Plasma, whole blood and erythrocyte potassium, red cell and plasma volume and erythrocyte counts of rats during the course of infection with* Trypanosoma brucei

Rat No.	Days after infection	Plasma K	Whole blood K	Red cell K	Red cell volume	Plasma volume	Trypanosome counts in thousands per cmm
		mg%	*mg%*	*mg%*	*%*	*%*	
1	0	18.7	194.5	386.8	47.5	52.5	0
	5	33.9	118.0	300.5	31.0	69.0	895
	Terminal	...	....	....	...	...	...
2	0	22.9	210.8	408.7	48.7	51.3	0
	5	47.5	142.0	325.4	34.0	66.0	850
	Terminal	40.8	93.6	214.9	30.2	69.8	950
3	0	19.8	209.9	400.0	50.0	50.0	0
	5	37.6	120.4	274.2	35.0	65.0	725
	Terminal	...	....	....	...	...	...
4	0	19.9	203.4	402.2	48.0	52.0	0
	5	...	...	...	...	...	...
	Terminal	55.8	101.8	206.6	30.5	69.5	965

For red cell volume determinations (hematocrit), Van Allen's (1925) method was employed. Blood was drawn by suction from the tail blood of rats to the 100 mark. An anticoagulant (1.3 per cent) solution of sodium oxalate was drawn up into the tube until the bulb was filled with the solution. After sealing the tube, it was centrifuged for 30 minutes or until all the cells were packed to a constant volume. The centrifuge was set at 3,000 revolutions per minute. The graduations at the top of the cell gave the reading directly in per cent. The plasma volume per cent used in the calculation also was obtained by subtracting the volume per cent of the hematocrit readings from 100. For counting the trypanosomes, the method described by Zwemer and Culbertson (1939) was employed.

RESULTS

The results of the experiments are shown in Tables 1 and 2. Table 1 gives the results of the plasma, whole blood and calculated erythrocyte potassium of rats infected with *T. brucei*. As the results obtained in previous investigations (Zwemer and Culbertson, 1939; Ikejiani, 1946), the plasma potassium increased and doubled by the time of death. In this experiment, the whole blood potassium consistently fell off and was reduced to approximately 33 to 48 per cent of the initial values during the terminal stages of infection. Erythrocyte potassium calculated on the basis of

TABLE 2.—*Plasma, whole blood and erythrocyte potassium, red cell and plasma volume and erythrocyte counts of rats during the course of infection with* Trypanosoma equiperdum

Rat No.	Days after infection	Plasma K	Whole blood K	Red cell K	Red cell volume	Plasma volume	Trypanosome counts in thousands per cmm
		mg%	*mg%*	*mg%*	*%*	*%*	
1	0	21.7	196.8	358.4	42.0	48.0	0
	5	29.5	132.5	301.9	37.8	62.2	740
	Terminal	49.2	93.7	238.6	23.5	76.5	1,200
2	0	19.9	185.9	456.7	38.0	62.0	0
	5	32.0	126.0	345.3	30.0	70.0	842
	Terminal	39.5	99.5	270.3	26.0	74.0	900
3	0	21.1	200.0	400.1	47.2	52.8	0
	5	...	....	....	...	...	...
	Terminal	53.7	118.0	301.9	25.9	74.1	750
4	0	22.3	185.0	391.7	44.1	54.9	0
	5	26.4	....	....	38.6	61.4	...
	Terminal	36.9	120.0	296.5	32.0	68.0	995
5	0	22.6	203.5	405.5	47.5	52.5	0
	5	42.0	109.0	....	...	...	...
	Terminal	40.1	102.0	228.8	32.8	67.2	1,220

hematocrit readings was reduced 46 to 49 per cent during the terminal stages of infection.

Table 2 shows the results of the whole blood, plasma and erythrocyte potassium determinations during infection with *T. equiperdum*. The plasma potassium increased as in infection with *T. brucei*. The whole blood potassium was reduced 25 to 53 per cent of the initial value while the erythrocyte potassium was similarly reduced 23 to 41 per cent during the terminal stages of infection.

The hematocrit readings reveal that in practically all the rats, the red cell volume was decreased. The terminal values showed a reduction of approximately 34 to 39 per cent. This seems to be in accord with the reduction in the number of the red blood cells due to anemia.

DISCUSSION

The increased serum potassium during infection with pathogenic trypanosomes is the result of the differential permeability of the red blood cells as shown by the investigations of Zwemer and Culbertson (1939). However, since the total plasma or serum potassium cannot all be accounted for as shown by the whole blood and erythrocyte potassium (Tables 1 and 2), the increased serum potassium is released from the body cells in general, due to generalized tissue cellular damage. Andrews, Johnson and Dormal (1930) and unpublished studies of Mautner and Ikejiani have shown that there are degenerative tissue changes particularly in the liver, spleen and kidney in pathogenic trypanosome infections. The pathological changes in the kidney, in particular suggest the possibility that the selective mechanism by which the kidneys aid in maintaining a constant blood plasma potassium must have been injured. Thus, this increase in plasma potassium as a result of generalized tissue cellular damage seems very nearly correct since any toxic or infectious agent that will affect the membrane permeability of the red blood cells will undoubtedly also attack other body cells in general with subsequent loss of potassium into the plasma.

Potassium has been shown to be toxic when injected into animals. Truszkowski and Zwemer (1938) have shown that the injection of potassium into animals causes an elevation of serum potassium toxicity. Emmens and Marks (1942) have also shown that intraperitoneal injection of 6.37 mg of potassium chloride per 10 gm body weight into young male albino rats caused 50 per cent mortality.

Not infrequently, however, it has been assumed that conditions existing in the blood at the onset or at the height of a disease were involved in the causation of death of the host. That this is so is evident from the different hypotheses which have been advanced in order to explain the pathogenesis of trypanosomiasis. These hypotheses have been reviewed by Taliaferro (1929) and von Brand (1938), and resolve themselves into one or another of two possibilities: death from hypoglycemia or from lactic acid accumulation. Recently, Zwemer and Culbertson (1939) have added increase in serum potassium as a possible cause of death in pathogenic trypanosome infection. But chemical changes in the blood during any disorder may be pathognomic without being pathogenic. To regard hypoglycemia, for instance, as a diagnostic sign of trypanosomiasis in the laboratory animals because this condition is present in the blood during the disorder is one thing; but to regard it as a responsible factor in the pathogenesis of the disease on the basis of the same data is quite another matter.

It is quite obvious that the increase in serum potassium observed in these experiments must have caused some toxicity. However, the serum potassium increase

was not shown in rats until the late stages of infection. Even at 24 hours before death, this increase in serum potassium was not significant. Such increase in serum potassium should have more effect in cases where distinctly high values were observed at least some days before death. An increased serum potassium occurring only a few hours before death should have little significance. It may not be the cause of death but rather a consequence of the moribund state.

It is of interest to note that similar increases in serum potassium have been observed in other types of disease and therefore are not specific for trypanosome infections. It is known, for instance, that potassium is elevated in Addison's disease. Zwemer and Scudder (1938) have observed serum potassium increase in shock. Zwemer, Sims and Coggeshall (1940) have shown that the plasma or serum potassium of man and animals increased during malaria infections. Scudder, Zwemer and Whipple (1938) have shown that in acute intestinal obstructions, potassium of the serum was increased.

In order, therefore, to estimate fully the rôle of potassium as a possible lethal factor in pathogenic trypanosome infections, it would not only be necessary but interesting to treat with drugs (tryparsamide, germanin) animals near death, which show an elevated potassium to see how long the potassium increase persists, and to find a drug to correct the altered blood chemistry, i.e., to immobilize potassium. These investigations are now being conducted by the author.

SUMMARY

Albino rats infected with pathogenic *Trypanosoma brucei* and *Trypanosoma equiperdum* show an increased serum potassium and decreased whole blood and erythrocyte potassium. There was also a decreased erythrocyte volume.

It is suggested that the serum potassium increase must have come from the body cells as a result of generalized tissue cellular damage.

Since potassium of the serum increased only at the terminal stages of infection it is suggested that potassium is not the cause of death but a consequence of the moribund state.

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STUDIES IN TRYPANOSOMIASIS.[1] IV. THE FRAGILITY OF THE ERYTHROCYTES IN RATS DURING THE COURSE OF INFECTION WITH *TRYPANOSOMA LEWISI, TRYPANOSOMA BRUCEI* AND *TRYPANOSOMA EQUIPERDUM*

OKECHUKWU IKEJIANI[2]
Department of Pathology and Bacteriology, University of Toronto

It has been shown by Dacie and Vaughan (1938) and Creed (1938) that there was an alteration in the fragility of the erythrocytes associated with variations in carbon dioxide content of the blood and that this was most marked in conditions of anemia. Tsai, Chen and Chiu (1943) showed that the rise of potassium levels ran almost parallel with the erythrocyte fragility *in vitro*.

Zwemer and Culbertson (1939) and Ikejiani (1946) showed that there was a marked rise in blood potassium of rats during infection with pathogenic trypanosomes. Ikejiani (1946) showed that such a rise was not observed in rats infected with non-pathogenic *T. lewisi*. Anemia has been observed in practically all investigations with pathogenic trypanosome infections. Andrews, Johnson and Dormal (1930) and Linton (1930) showed that in pathogenic trypanosome infections there was a marked acidosis which began during the early stages. Such was not the case with non-pathogenic *T. lewisi* infection.

In view of the interesting observations of Dacie and Vaughan (1938), Creed (1938), Tsai, Chen and Chiu (1943), Andrews, Johnson and Dormal (1930) and Linton (1930), a study was made of the resistance of the erythrocytes of rats, infected with pathogenic *T. brucei* and *T. equiperdum* and non-pathogenic *T. lewisi,* to hemolysis by hypertonic salt solution.

METHOD

Three groups of rats were infected with *T. lewisi, T. brucei* and *T. equiperdum* respectively. Blood samples were withdrawn by heart puncture from each rat prior to infection and the erythrocytic fragility test made. This was taken as the normal sample. The animals were then infected and blood samples were obtained at intervals thereafter for erythrocytic fragility tests, until death in those rats infected with pathogenic trypanosomes, or until the trypanosomes had practically disappeared from the blood stream in cases of those rats infected with non-pathogenic *T. lewisi.* The erythrocytic fragility towards hypertonic saline solution was determined by the method of Creed (1938). Trypanosome counts were made during each set of fragility tests according to methods described by Zwemer and Culbertson (1939).

RESULTS

As shown in the tables two stages in the process of hemolysis were noted in these experiments, namely, the point of commencing hemolysis and the point of complete

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TABLE 1.—*The fragility test of the erythrocytes and trypanosome counts of rats during the course of infection with* Trypanosoma lewisi

Rat No.	Determinations	Normal test before infection %	Samples tested at designated days (given in per cent), after infection				
			3	8	16	20	32
1	Fragility	0.26–0.22	0.28–0.24	0.26–0.24	0.26–0.22	.....	0.26–0.24
	Trypanosome*	0	+	195	50	.....	0
2	Fragility	0.26–0.22	0.26–0.22	0.28–0.26	0.28–0.24	.....	0.26–0.22
	Trypanosome	0	+ +	150	65	.....	0
3	Fragility	0.28–0.24	.....	0.30–0.34	0.28–0.24	0.28–0.22	0.26–0.24
	Trypanosome	0	.....	100	40	25	.....
4	Fragility	0.30–0.24	.....	0.30–0.24	.....	0.28–0.24	.....
	Trypanosome	0	.....	85	.....	100	.....
5	Fragility	0.28–0.22	0.24–0.24	.....	0.28–0.24	0.28–0.22	0.28–0.24
	Trypanosome	0	+ +	.....	25	50	.....
6	Fragility	0.26–0.20	0.28–0.24	0.28–0.24	0.26–0.20	.....	0.26–0.22
	Trypanosome	0	+	120	450	.....	.....

* Key to figures in tables : 0, no trypanosomes ; +, between one and five trypanosomes per cmm tail blood ; ++, between five and ten trypanosomes per cmm tail blood ; 40, 150, 1,012, etc., number of trypanosomes in thousands per cmm tail blood.

hemolysis. Table 1 shows the results of the fragility of the erythrocytes of rats infected with *T. lewisi.* No significant change was observed in the resistance of the erythrocytes to hemolysis by hypertonic salt solution. Even during the height of infection the slightly diminished resistance of the erythrocytes to hemolysis was not significant.

Table 2 and 3 show the results of the fragility of the erythrocytes of rats infected with *T. brucei* and *T. equiperdum,* respectively. In both of these infections, the fragility of the erythrocytes showed a consistent increase, that is, there was a consistent diminished resistance of the erythrocytes to hemolysis by hypertonic salt solution. Such tendency to hemolysis was more marked as the infection progressed.

DISCUSSION

Two factors have been proven to cause diminished resistance or increased fragility of the erythrocytes to hemolysis by hypertonic salt solution. These factors are acidosis associated with anemia and increased blood potassium. These conditions have been shown to exist in pathogenic trypanosome infections but not in non-

TABLE 2.—*The fragility test of the erythrocytes and trypanosome counts of rats during the course of infection with* Trypanosoma brucei

Rat No.	Determinations	Normal test before infection %	Samples tested at designated days after infection in %				
			2	3	4	5	Terminal
1	Fragility	0.28–0.24	0.30–0.28	.....	0.42–0.36	.....	0.48–0.38
	Trypanosome*	0	+ +	.....	750	.....	900
2	Fragility	0.22–0.20	0.28–0.26	0.32–0.26	.....	.....	0.40–0.32
	Trypanosome	0	+	200	.....	.....	1,070
3	Fragility	0.28–0.20	0.28–0.26	.....	0.34–0.30	0.42–0.38	.....
	Trypanosome	0	+	.....	500	700	.....
4	Fragility	0.22–0.22	0.26–0.24	0.34–0.30	.....	.....	0.44–0.34
	Trypanosome	0	+ +	150	.....	.....	980
5	Fragility	0.24–0.20	0.28–0.28	.....	0.40–0.34	.....	0.40–0.32
	Trypanosome	0	+ +	.....	600	.....	1,012
6	Fragility	0.30–0.24	0.32–0.36	0.36–0.36	.....	0.48–0.38	0.46–0.36
	Trypanosome	0	+ +	190	.....	650	800
7	Fragility	0.28–0.26	0.30–0.30	0.36–0.38	0.44–0.40	0.44–0.34	0.48–0.40
	Trypanosome	0	+ +	300	650	900	750
8	Fragility	0.30–0.30	0.32–0.28	0.34–0.32	0.38–0.32	.....	0.40–0.34
	Trypanosome	0	+ +	200	400	.....	1,200

* See footnote, Table 1.

TABLE 3.—*The fragility test of the erythrocytes and trypanosome counts of rats during the course of infection with* Trypanosoma equiperdum

Rat No.	Determinations	Normal test before infection %	Samples tested at designated days after infection in %				
			2	3	4	5	Terminal
1	Fragility	0.26–0.24	0.30–0.36	.....	0.38–0.34	0.42–0.38	.....
	Trypanosome*	0	+	.....	640	800	.....
2	Fragility	0.26–0.22	0.26–0.24	.....	0.38–0.32	.....	0.40–0.38
	Trypanosome	0	+ +	.....	400	.....	700
3	Fragility	0.22–0.22	0.26–0.22	.....	0.32–0.32	0.38–0.32	0.38–0.34
	Trypanosome	0	+ +	.....	700	870	1,000
4	Fragility	0.24–0.24	0.28–0.24	0.36–0.36	0.34–0.30	.....	0.40–0.32
	Trypanosome	0	+ +	250	640	.....	900
5	Fragility	0.24–0.22	0.30–0.28	0.34–0.32	.....	0.38–0.38	.....
	Trypanosome	0	+ +	190	.....	600	.....
6	Fragility	0.28–0.24	0.28–0.26	.....	.....	.....	0.42–0.36
	Trypanosome	0	+	.....	.....	.....	950
7	Fragility	0.26–0.22	0.28–0.24	0.30–0.30	0.42–0.36	0.48–0.38	0.40–0.34
	Trypanosome	0	+	200	750	940	1,200
8	Fragility	0.26–0.24	0.32–0.26	0.38–0.32	.....	0.44–0.40	.....
	Trypanosome	0	+ +	140	.....	1,150	.....

* See footnote, Table 1.

pathogenic trypanosome infections (Zwemer and Culbertson, 1939; Linton, 1930; and Andrews, Johnson and Dormal, 1930). These two factors, therefore, are responsible for the diminished resistance or increased fragility of the red blood cells of rats infected with *T. brucei* and *T. equiperdum*. With the exception of slight anemia, such factors are not present in *T. lewisi* infection, and consequently no significant change in erythrocytic fragility was observed in this infection.

However, since acidosis associated with anemia has been shown to begin early during infection with pathogenic trypanosomes, it may be assumed that acidosis is the first and prominent cause of diminished resistance or increased fragility of the erythrocytes to hemolysis by hypertonic salt solution. In no way does this minimize the fact that increased blood potassium in these infections is a contributory factor at the late stages of infections to the diminished resistance of the erythrocytes.

SUMMARY

There was an increased fragility or diminished resistance to hypertonic salt solutions of the erythrocytes of rats infected with pathogenic *T. brucei* and *T. equiperdum*. No such results were obtained with the erythrocytes of rats infected with non-pathogenic *T. lewisi*.

Acidosis associated with anemia which begins early during infection with pathogenic trypanosome infection has been assumed to be the cause of this diminished resistance of the erythrocytes although increased blood potassium during the late stages of infection might also be a contributory factor. These factors explain the difference in the fragility of the erythrocytes observed in rats infected with *T. brucei* and *T. equiperdum* on the one hand, and *T. lewisi* on the other.

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STUDIES ON THE LIFE HISTORY AND DEVELOPMENT OF *MACRACANTHORHYNCHUS INGENS* MEYER, 1933, WITH A REDESCRIPTION OF THE ADULT WORM[1]

DONALD V. MOORE[2]
Senior Parasitologist, Bureau of Laboratories, Texas State Health Department

The adult *M. ingens* used in these studies were recovered from raccoons (*Procyon lotor lotor* L.) trapped in Angelina County, Texas (Chandler, 1942). A total of thirteen raccoons were examined and eleven of them were found infected with *M. ingens*. Up to ninety mature and immature ACANTHOCEPHALA were recovered from a single raccoon.

REDESCRIPTION OF *Macracanthorhynchus ingens*

This worm was first described by von Linstow (1879) as *Echinorhynchus ingens*. Travassos (1917) placed it in the genus *Prosthenorchis*. Meyer (1933) removed it from *Prosthenorchis* and included it in the genus *Macracanthorhynchus* on the basis of the large size of the body and the nature of the outer shell of the egg. Meyer's description of the species, taken from von Linstow's original description, contains only the following meager data: Body 145 mm long, 3 mm wide, irregularly wrinkled. Proboscis with 6 rows of hooks (transverse rows), hooks 0.23 mm long. Eggs 0.098 mm long, 0.056 mm wide, dark-brown in color and ellipsoidal in shape and have a double shell the outer of which is characterized by a fine network of ridges. Recorded from *Procyon lotor lotor* L.

Since this description is inadequate in many respects, the following more extended description is given:

Macracanthorhynchus ingens (von Linstow, 1879) Meyer, 1933
Synonyms: *Echinorhynchus ingens* von Linstow, 1879
Prosthenorchis ingens (v.L.) Travassos, 1917

Mature females 183 to 300 mm in length and 5 to 8 mm in width. Mature males 130 to 150 mm by 4 to 5 mm. Proboscis with sub-globular extremity at end of unarmed basal region. Hooks limited to sub-globular extremity (Fig. 10). Proboscis 0.9 to 1.0 mm in length, sub-globular region 0.58 to 0.6 mm in length, basal region 0.33 to 0.41 mm in length. Maximum diameter of sub-globular portion 0.70 to 0.84 mm, of basal portion 0.54 to 0.66 mm. Proboscis hooks arranged in 6 circular rows of six hooks each; total number of hooks 36. Hooks of three distinct types, designated Types I, II, III. Type I (Fig. 11) includes hooks of first two circular rows, slightly recurved, with simple point, and provided with long anteriorly directed root and a short posteriorly directed root; average outside measurement, including anterior root, 24.5 microns; average length of hook from tip to base of anterior root, 15.2 microns; average length of anterior root, 9.3 microns. Type II (Fig. 12) in third and fourth circular rows, more strongly recurved than Type I, with chisel-shaped point, and provided with shorter anterior root; average outside measurement, including anterior root, 15 to 18 microns; length of hook, 12 to 14 microns; anterior root, 3 to 4 microns. Type III (Fig. 13) in fifth and sixth circular rows, small, with simple point, only slightly recurved; anterior and posterior roots reduced to mere protuberances; 7 to 8.4 microns in length.

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Testes, two, elongate, arranged in tandem in middle of body about 11 mm apart, measuring 11 to 12 mm by 0.91 to 0.96 mm. Eight cement glands, ellipsoidal in shape (Fig. 14).

Mature eggs (Fig. 1) elliptical in shape and provided with heavy, dark-brown outer shell, irregularly grooved or ridged. Inner shell thick and highly refractive. Between outer and inner shells a thin elastic membrane. Acanthor, with rostellum invaginated, confined within limits of inner shell, surrounded by thin elastic membrane. Outer shell 96 to 106 microns long, 51 to 54 microns wide with mean of 97 by 52 microns. Inner shell, 3 to 6 microns thick, 67 to 71 microns long, 32 microns wide, with mean of 69 by 32 microns. Acanthor within egg shells 61 to 64 microns long, 25 to 26 microns wide.

The measurements in the above description were made on fully mature, adult worms. It was noted that some variation occurs in proboscis measurements made on immature worms.

LIFE HISTORY STUDIES

There have been no previous published reports concerning the life cycle of *Macracanthorhynchus ingens,* although the life history of another member of the genus, *M. hirudinaceus,* was first discovered by Schneider (1871) and its development in different intermediate hosts was studied by Kaiser (1887, 1893) and later by Meyer (1931, 1933, 1936, 1938). Kates (1943) has made a study of the development of *M. hirudinaceus* in certain intermediate hosts common in the United States.

MATERIALS AND METHODS

The eggs used in the experimental life history studies were from two sources; the body cavity of gravid female worms, and the contents of the large intestine of infected raccoons.

The larvae of scarabaeid beetles used as experimental intermediate hosts were collected from gardens in the residential district of Houston, Texas, and from the Rice Institute Campus. Examination of representatives of each collection of grubs showed them to be free of natural ACANTHOCEPHALA infections. The grubs in which successful experimental infection was attained were *Phyllophaga crinita* Burmeister, *Phyllophaga hirtiventris* Horn, and *Ligyrus sp.*

Since the grubs have a tendency to be pugnacious and cannibalistic when overcrowded, they were cultured individually in small clay flower pots filled with a medium made up of one part of sterilized manure and five parts of garden soil. In order to prevent desiccation the pots were placed in a flat metal tray, the bottom of which was covered with several layers of paper towels which were periodically saturated with water.

Before the grubs were infected, the soil was loosened and the grub dug up. The infective material, composed of a high concentration of eggs in about 5 cc of water, was evenly distributed over the surface of the soil. The eggs were washed deeper into the soil by the repeated application of water; this may be extended over two or three days.

The microscopical techniques were the same as those used in previous studies (Moore, 1946).

GENERAL LIFE CYCLE

The method of infecting the grubs made it impossible to determine the exact age of the ACANTHOCEPHALA larvae recovered. Consequently, a range of 5 to 10 days is given for the appearance of definite larval stages. The age given refers to the number of days after the infection was administered and may not be the actual age of the larva.

After ingestion, fully embryonated eggs hatch in the mid-gut. Following hatching, the torpedo-shaped acanthor penetrates the gut wall by the retraction and extension of its rostellar hooks, accompanied by the contraction and elongation of its body. As the acanthor progresses through the gut wall, it begins to grow, loses its characteristic torpedo-shape, and becomes more or less oval. In thirty to thirty-five days the larva appears on the exterior of the gut wall as a small, spherical, iridescent speck. A few days later it drops into the haemocoele and comes to lie among the fat bodies. In the haemocoele the larva elongates and the inner nuclear mass differentiates into the primordia of the various adult structures. When a particular grub has a heavy infection with *M. ingens* larvae, the elongate larvae may be seen through the dorsal surface of the thin portion of the body wall covering the hind gut (Text figure). The pre-acanthella makes its appearance in 50 to 55 days. The acanthella may be found encysted in the haemocoele 60 to 70 days after the administration of the infection.

The acanthella remains in a quiescent state until the grub is ingested by a transport host or the final host. It persists through the transformation period of the grub into the adult beetle without losing its infectivity. When it reaches the intestinal tract of a transport host, such as a frog, it is freed from its cyst and penetrates the intestinal wall into the coelom, where it may wander around for several days before re-encystment in the mesenteries or peritoneum. The temperature of the soil in which the grubs were cultured was 20° to 22° C and the rate of development shown in these experiments refers to the devlopment at this temperature.

DESCRIPTION OF DEVELOPMENTAL STAGES

Egg (Fig. 1)

The egg or shelled embryo of *Macracanthorhynchus ingens* is described above. The thick, dark-brown outer shell of the egg is covered by a very thin membrane which may be lost before the egg leaves the body cavity of the female worm or shortly thereafter, so that eggs from the droppings of infected animals seldom if ever possess this membrane.

Acanthor (Figs. 2 and 3)

The eggs of *M. ingens* may be hatched artificially according to the technique described by Manter (1928) with improvements by Moore (1942). Upon rewetting the eggs in dried smears the heavy outer shell usually splits at the anterior pole of the egg and the split continues in an irregular line along the longitudinal axis of the egg. The outer shell seldom becomes completely detached from the middle embryonic enevelope. The inner shell usually splits at the anterior pole and acanthor is mechanically forced out by its increase in size due to the absorption of water. Under natural conditions in the gut of the grub, the acanthor probably plays an active part in escaping from the egg.

The artificially hatched acanthor is torpedo-shaped, tapering toward the posterior end, and measures 139 to 155 microns by 29 to 41 microns, the width being measured at a point about midway between the anterior and posterior ends. In the fully extended acanthor the rostellum is not definitely set off from the rest of the body, and its posterior limitation can only be determined externally by the greater size of the rostellar spines as compared with the smaller body spines, and internally by the beginning of the central nuclear mass.

The rostellum is armed with three pairs of blade-like hooks; one medial pair and two lateral pairs of unequal size, and four rows of rostellar spines. The two median

TEXT FIGURE. Encysted acanthellas of *M. ingens* in the haemocoele of an experimentally infected grub of *Phyllophaga crinita*.

rostellar hooks originate from the apex of the rostellum and extend outward, being connected with the rostellum except for about 3 microns at the free tip. They measure 15 to 16 microns in length and are about 4 microns wide at the widest point,

which is about 3 microns from the free tip. The larger pair of lateral hooks, adjacent to the median hooks, measure 12 to 13 microns in length and have a maximum width of 2 to 3 microns. The smaller outer pair of lateral hooks are 9 to 11 microns long and very narrow. The two pairs of lateral rostellar hooks do not take origin from the exact center of the rostellum.

The first row of rostellar spines consists of two pairs, each pair being located between the lateral hooks of opposite sides near the center of the rostellum. These are larger and wider than the rest of the rostellar spines. The remaining three alternating rows of rostellar spines possess 16 to 18 spines in each row. Those of the third row are smaller and grade into the minute cuticular spines of the body, which are arranged in 18 to 20 alternating circular rows, with 16 to 18 spines in each row toward the anterior end of the body, diminishing to 8 to 12 in each row toward the posterior end of the body.

The musculature of the acanthor consists of two retractors and a circular muscle band associated with the rostellum, and a network of subcuticular muscles in the body wall. The two retractor muscles originate in the body wall about one-third of the way back toward the posterior end, and insert together in the apex of the rostellum at the base of the two median hooks. The subcuticular muscles of the body are made up of a network of thin muscle bands running longitudinally with a slightly clockwise spiral.

The central nuclear mass, 9 to 10 microns wide and made up of a band of closely packed undifferentiated nuclei, is located in the center of the acanthor, beginning at the base of the rostellum and extending posteriorly for about 50 microns, where it ends abruptly. The width and concentration of nuclei remain fairly constant throughout the length of the band. A few scattered vesicular subcuticular nuclei are located in the parenchymatous tissue beneath the subcuticular muscles.

CHANGES TAKING PLACE IN THE DEVELOPMENT FROM THE ACANTHOR TO THE PRE-ACANTHELLA

The acanthor penetrating the gut tissue of the grub retains its characteristic torpedo-shape for about 10 to 15 days (Fig. 3). Its diameter at this time is 20 to 23 microns, measured from sections of the acanthor in gut tissue. The nuclei of the central nuclear mass have enlarged slightly and the compactness of the mass has broken up to some extent. Between 15 and 20 days the acanthor becomes oval in shape and begins to lose its motility.

Between 20 and 25 days after infection (Fig. 4) most of the larvae in the gut tissue measure 180 by 130 microns. The larva is now divided into two distinct layers, a thick dermal layer and an inner cell mass. The dermal layer is characterized by the presence of large vesicular nuclei. It is believed that these nuclei have migrated from the inner nuclear mass into the parenchyma of the dermal layer. The inner nuclear mass measures 113 by 71 microns. Its nuclei have become larger, more vesicular, and outlined by definite membranes. The space between the nuclei is occupied by a finely-granular, compact parenchymatous tissue which usually stains much darker than the parenchyma of the dermal layer.

The combined primordia of the proboscis, proboscis receptacle and brain are recognizable at the anterior end of the inner cell mass. This structure appears as a rounded mass of tissue containing 3 or 4 nuclei and is 19 microns in diameter.

About thirty to thirty-five days after infection the larvae begin to elongate, and further differentiation in the inner nuclear mass occurs. The primordium of the brain separates from the spherical mass of tissue designated above. The region anterior to the brain will become the proboscis and the tissue lateral and posterior to it will become the proboscis receptacle. The solid mass of nuclei posterior to the primordium of the proboscis receptacle splits in its median longitudinal axis to form the body cavity.

Forty to forty-five days after infection the larva (Fig. 5) becomes more elongate and further differentiation of the proboscis region occurs. The brain is now a rounded compact mass of nuclei situated within the posterior region of the proboscis receptacle. Anterior to the brain the inverter muscles of the proboscis appear as two strands of tissue extending anteriorly to a group of nuclei at the apical end of the future proboscis. The developing proboscis is in contact with the dermal layer anteriorly but has separated from it laterally. Posterior to the proboscis receptacle the median longitudinal split has extended to one-half to three-quarters of the length of the inner nuclear mass. The nuclei in this region have become smaller and more numerous. The lacunar system develops as a series of fine transverse striations encircling the larva in the dermal layer immediately outside the body cavity. The nuclei of the dermal layer have become more evenly dispersed, and migration from the inner nuclear mass ceases. Larvae of this age are from 462 to 756 microns in length and 252 to 322 microns in width.

Between 45 and 50 days after infection the larvae continue to increase in length and the longitudinal split in the inner nuclear mass reaches the posterior end, completing the formation of the body cavity and ligament sacs. A small group of nuclei remains at the posterior end of the body cavity, later giving rise to the genital primordia. There are two ligament sacs, one dorsal and one ventral. In cross section they appear as two thin membranes attached to the body wall and in contact with each other in the center of the worm. Several small nuclei are found between the membranes of the two sacs, and in toto mounts this region appears as the genital ligament, extending from the base of the proboscis receptacle to the urogenital apparatus. Anteriorly the two sacs communicate with each other and their membranes separate from the body wall and attach to the base of the proboscis receptacle. The two lateral three-cornered spaces between the dorsal and ventral ligament sacs are regarded as the body cavity proper, which contains some of the nuclei remaining after the body cavity has been formed by delamination. These nuclei arrange themselves to form two bands on opposite sides of the body, which decrease in width posteriorly and are bifurcated anteriorly. The anterior ends of the bifurcations reach the level of the base of the proboscis receptacle. The nuclei in these bands will later take part in the development of the circular muscles in the body wall of the adult worm. The developing proboscis grows anteriorly into the dermal layer, and hooks begin to appear as thin, highly refractive lines extending a short distance into the dermal layer. The region of the proboscis receptacle posterior to the brain elongates in such a manner that the brain becomes located eccentrically in the cavity of the receptacle. During the latter part of this period of development (45 to 50 days) the testes take form as oval masses of tissue located in the genital ligament. In living material an outer hyaline sheath closely adherent to the larva can be observed.

Pre-acanthella (Figs. 6 and 7)

The pre-acanthella usually appears between 50 and 55 days after infection, and is developed by the continued elongation of the larva. The pre-acanthella measures 2.1 to 3.2 mm in length depending upon the stage of development. The greatest width is usually slightly anterior to the middle of the body length; it ranges from 0.36 to 0.54 mm. At this point the dermal layer ranges from 0.04 to 0.08 mm in thickness. The width of the body tapers gradually to 0.27 mm at the posterior end. In the region of the lemniscal ring the pre-acanthella is 0.47 to 0.54 mm in width. The combined length of the proboscis and proboscis receptacle varies from 0.47 to 0.64 mm. The region of the proboscis anterior to the lemniscal ring measures 0.25 to 0.36 mm in length. The total length of the proboscis varies from 0.32 to 0.40 mm. The central portion of the proboscis is hollow. The proboscis receptacle, inserted near the anterior tip of the proboscis, is 0.11 mm wide at the region of the brain. The inverters pass posteriorly through the center of the hollow proboscis and around the brain to the mid-ventral wall of the receptacle, where a portion of each becomes fused with the wall of the receptacle. The remainder of each band emerges at this point to become the retractors of the proboscis, which fuse with the body wall posterior to the receptacle. Small nuclei are evident in the inverters and retractors.

In the more fully developed pre-acanthella the proboscis is more globular, the hooks are slightly recurved and take on the general appearance of the hooks on the proboscis of the adult worm. The presence of each hook beneath the cuticula produces a slight protuberance on the proboscis.

The lemniscal ring is composed of 14 nuclei. During the latter part of the pre-acanthella stage two lemniscal buds develop extending into the body cavity on a level with the brain (Fig. 7). Seven nuclei of the lemniscal ring migrate into each bud. The apical ring composed of six small nuclei is located at the tip of the proboscis between the roots of the hooks of the first circular row.

The pre-acanthella is enveloped in a clear hyaline sheath, in which the larva usually lies straight.

Acanthella (Figs. 8 and 9)

From 55 to 60 days after infection the pre-acanthella continues to elongate, and the proboscis becomes more globular in shape and more distinctly set off from the body of the larva anterior to the lemnisci. Accompanying the elongation of the larva there is a flattening and an increase in width. The hypodermal nuclei lose their characteristic spherical shape and eventually disappear from view, becoming incorporated in the developing body wall. The proboscis hooks become larger and more heavily chitinized. They remain just beneath the cuticula of the proboscis for a while, eventually breaking through to the exterior. At this stage in development, the proboscis remains extended since its musculature and that of the receptacle are not functional. The inner layer of the proboscis receptacle becomes very muscular by the development of transverse muscle fibers. On the ventral side of the receptacle in the region of the brain a distinct cleft appears where the inverters of the proboscis emerge and become the retractor muscles.

The mature encysted acanthella can be recovered from the haemocoele of the grub (Text figure) 60 to 70 days after infection. The invagination of the proboscis into the body cavity differentiates this stage from the non-infective larvae of approximately the same size. Enveloping the acanthella is a hyaline fusiform cyst measur-

ing 2.3 to 3.2 mm in length by 0.9 to 1.05 mm in width. The cyst tapers toward each rounded end. Living specimens within the cyst are capable of extending and contracting the body as well as the proboscis. When the acanthella in the cyst is fully extended it completely fills the cyst, even stretching it to some extent. The fully extended acanthella removed from the cyst, fixed and mounted, measures 4.06 to 5.03 mm by 0.8 to 1.0 mm, tapering to 0.2 mm in width at the posterior end. The body wall of the acanthella is fairly uniform in thickness.

The proboscis and proboscis receptacle have reached their full development as far as the larval stages are concerned. The proboscis measures 0.56 to 0.60 mm in length and 0.50 to 0.56 mm in maximum diameter. The hooks are restricted to the sub-globular extremity. The unarmed neck region of the proboscis measures 0.23 mm in length by 0.3 to 0.4 mm in width. The above proboscis measurements were made on fixed, stained specimens. The proboscis receptacle has developed thick muscular walls. The receptacle is smaller in diameter posterior to the cleft where the retractors of the proboscis emerge. The brain is eccentrically located on a level with the cleft. This heavy muscular portion of the receptacle is 0.7 to 1.2 mm in length. The diameter of the receptacle is 0.25 to 0.30 mm anterior to the cleft, and 0.18 to 0.20 mm posterior to it.

The lemnisci have become very long, usually extending to the posterior tip of the body cavity. In the fully extended condition the lemnisci are longer than the body cavity; this necessitates curving and folding.

The two bands of small nuclei previously described as being located on opposite sides of the body cavity and bifurcated at the anterior end are still present in young acanthellas. In cross sections, processes from these nuclei can be seen extending into the parenchyma of the body wall, where eventually they become incorporated in the development of the body musculature.

The testes are sub-ovate in shape measuring 0.18 to 0.2 mm by 0.07 to 0.09 mm. They are located in tandem in the genital ligament in the posterior region of the body, and are quite widely separated. The eight undeveloped cement glands can be observed as a group of very small nuclei between the testes and the seminal vesicle. The ovaries consist of small masses of cells free in the body cavity or attached to the genital ligament.

POSSIBLE TRANSPORT HOST

The raccoon is generally omnivorous in its feeding habits; frogs, fish, reptiles, insects and shell fish are the more important animal foods. Ernest Thompson Seton in his "Life Histories of North American Mammals" states that under natural conditions during the summer months the raccoon feeds mainly on frogs. Consequently, an experiment was performed to determine whether the frog plays a significant rôle in the completion of the life cycle of *Macracanthorhynchus ingens*.

One frog (*Rana pipiens*) was given 10 acanthellas from the haemocoele of one of the experimentally infected grubs. Three days later one of the acanthellas was passed in the droppings of the frog, devoid of its cyst but apparently unharmed. Twelve days after the administration of the infection, the frog was killed and autopsied. One acanthella was found encapsulated in the mesentery in the region of the duodenum, and three were found wandering in the peritoneal cavity. No evidence of growth was noted in any of the larvae recovered from the frog.

It is evident from this that the frog, though not an essential intermediate host, may act as a transport host. Since the acanthellas persist through the transformation of the grubs into adult beetles, the frogs might acquire this parasite in nature by feeding on infected beetles. It is probable, however, that the raccoons can acquire the infection directly by feeding on either grubs or adult beetles.

DISCUSSION

In comparing the life history and development of *M. ingens* and *M. hirudinaceus* a great many similarities are observed. The basic pattern of development is the same. The intermediate hosts used by both parasites are the larvae of scarabaeid beetles, but neither worm appears to be limited to a particular species of scarabaeids. A search of the literature reveals that over twenty-five species of grubs, scarabaeids, carabaeids, and other coleopterous larvae living under similar conditions can serve as intermediate hosts for *M. hirudinaceus*. Further investigation would probably reveal that *M. ingens* is just as non-specific. The total time required for the development of the acanthella of *M. hirudinaceus* ranges from 60 to 90 days according to Kates (1943). *M. ingens* requires from 60 to 70 days to complete its development. This is not a significant variation since the rate of development is dependent on the environmental temperature of the intermediate host.

In view of present available data there seems to be very little significant difference between the acanthor stage of the two species. There is some variation in the number of rows of body spines, and some difference in size. Whether or not these are significant differences is uncertain.

The developmental stages between the acanthor and the pre-acanthella can be designated only by age and therefore it is not practical to use them for comparisons except in the basic developmental pattern.

Kates (1943) has shown that the pre-acanthella of *M. hirudinaceus,* which he designates as a Stage IV acanthella, makes its appearance between forty and forty-five days. The pre-acanthella of *M. ingens,* according to the author's experiments, appears between fifty and fifty-five days. Both species have the characteristic bifurcated bands of body muscle nuclei in this stage. Kates (1943) and other investigators have shown that *M. hirudinaceus* has 12 nuclei in the lemniscal ring, and 6 nuclei in each lemniscus. *M. ingens* has 14 nuclei in the lemniscal ring and 7 nuclei in each lemniscus. This seems to be a constant characteristic and future investigators may show it to have phylogenetic significance.

There seems to be little difference except in measurement between the infective acanthellas of the two species. *M. ingens* may use a transport host such as *Rana pipiens* to complete the life cycle. There seems to be no available information regarding the use of a transport host by *M. hirudinaceus*.

SUMMARY

The life history and development of *M. ingens* has been determined by experimental infections and complete descriptions of the developmental stages given. The intermediate hosts so far determined are grubs of the scarabaeid beetles: *Phyllophaga crinita, Phyllophaga hirtiventris,* and *Ligyrus sp.*

The period of development at temperatures of 20 to 22° C varies from 60 to 70 days. Penetration of the gut wall of the intermediate host occurs 30 to 35 days

after infection. The pre-acanthella develops 50 to 55 days, and the infective acanthella 60 to 70 days after infection.

In one experiment a frog was shown to be a possible transport host. It does not seem probable that the transport host is necessary for the completion of the cycle, but no experimental infections of the definitive host were attempted.

Some comparisons are drawn between the development of *M. hirudinaceus* and *M. ingens*.

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Explanation of Plates

All figures were drawn with the aid of a camera lucida

Abbreviations Used

AP—apical papilla
B—brain or ganglion
C—cyst
CNM—central nuclear mass
DL—dermal layer
GL—genital primordia
HT—host tissue
I—inverters
ICM—inner cell mass
IS—inner shell
L—lemnisci
MC—muscle cells
N—giant nuclei of dermal layer
NA—nuclei of apical ring
NL—nuclei of lemniscal ring
OS—outer shell
PA—proboscis primordium
PR—proboscis receptacle
RH—rostellar hooks
RM—retractor muscles
RS—rostellar spines
T—testes

Plate I

Fig. 1. Mature egg containing acanthor (drawn in optical section).
Fig. 2. Acanthor (artificially hatched).
Fig. 3. Median sagittal section of acanthor in gut wall of the grub of *Phyllophaga crinita,* 15 days after the exposure of the grub to infection.
Fig. 4. Median sagittal section of the larva in the gut wall of the grub of *Phyllophaga crinita,* 25 days after infection.
Fig. 5. Larva from the haemocoele of the grub of *Phyllophaga hirtiventris,* 43 days after infection.
Fig. 6. Pre-acanthella (male) from haemocoele of the grub of *Phyllophaga hirtiventris,* 50 days after infection.
Fig. 7. Pre-acanthella (female) from haemocoele of the grub of *Phyllophaga crinita,* 55 days after infection.

Plate II

Fig. 8. Acanthella (male) with the proboscis evaginated and freed from its enveloping cyst (from the haemocoele of *Phyllophaga crinita*).
Fig. 9. Encysted acanthella with proboscis invaginated, from haemocoele of *Phyllophaga hirtiventris* (drawn from living material).
Fig. 10. Proboscis of the acanthella.
Fig. 11. Type I proboscis hook.
Fig. 12. Type II proboscis hook.
Fig. 13. Type III proboscis hook.
Fig. 14. Posterior end of adult male worm showing cement glands and genital apparatus.

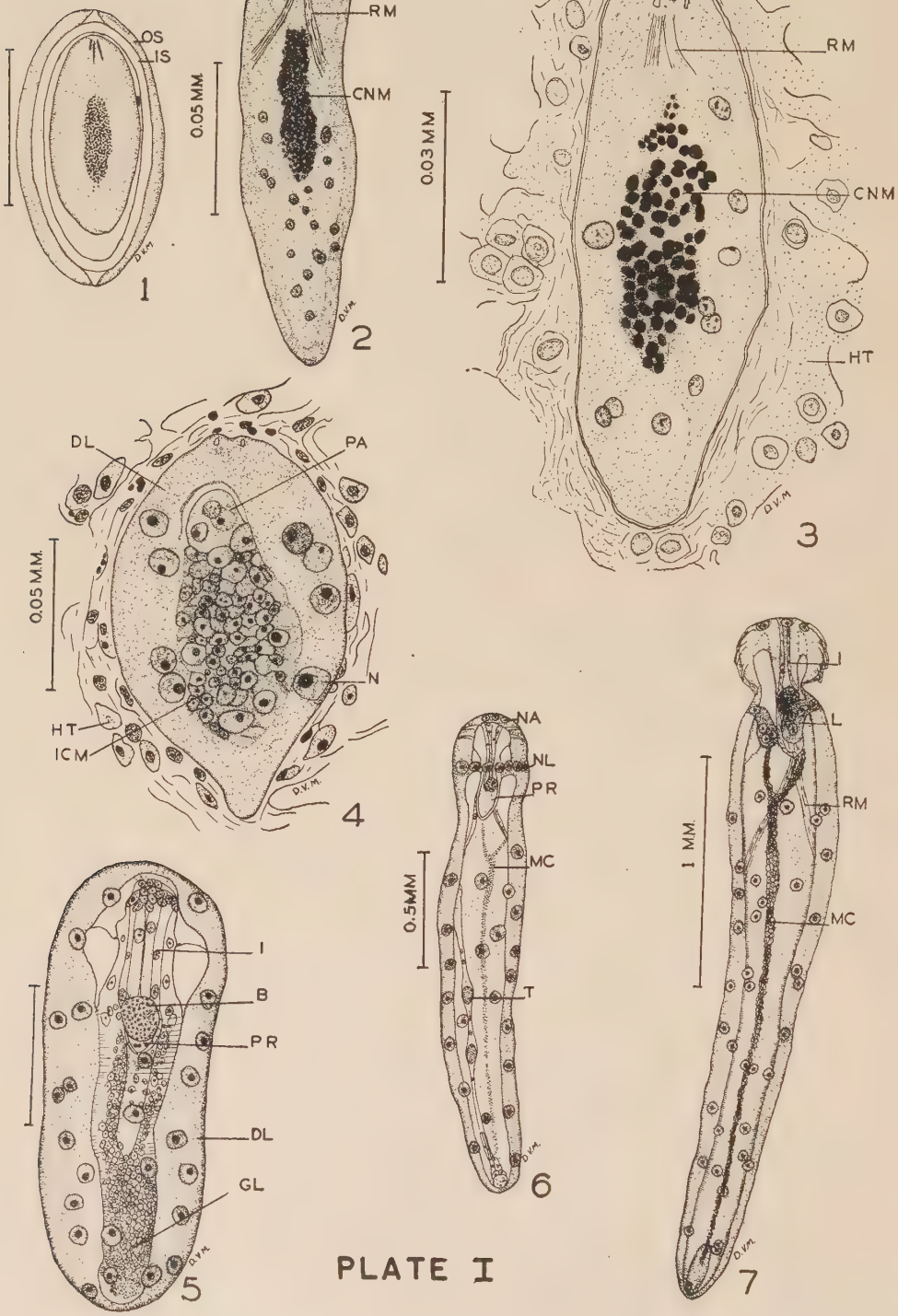
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PLATE I

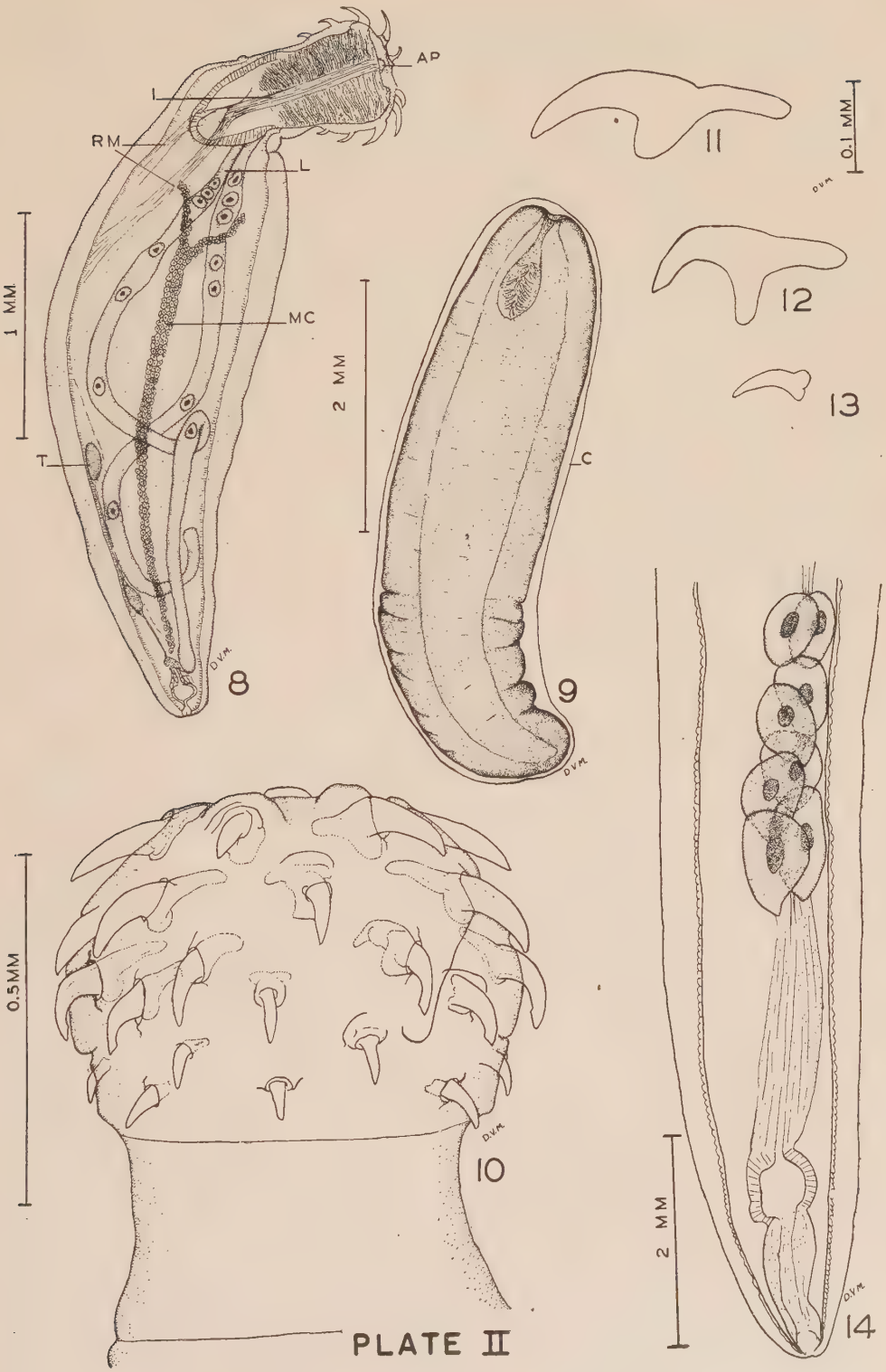

PLATE II

SPECIFIC SOLUBLE ANTIGEN IN THE BLOOD OF FILARIAL PATIENTS

Lt. Comdr. M. B. Franks

Medical Corps, United States Naval Reserve,[1] United States Naval Medical Research Unit No. 2[2]

In recent tests, Franks, Chenoweth and Stoll (1946), the reactivity of skin-test antigens prepared from larvae as well as from adult worms of *Dirofilaria immitis* was compared in persons, chiefly Okinawans, infected with *Wuchereria bancrofti*. The dermal responses were so varied and intriguing that the present study was initiated in an effort to explain their mechanisms. In the work already reported, Franks et al (1946), the skin reactions elicited by microfilarial and adult-worm antigens appeared to be influenced or altered by the clinical phase of the disease. However, there was no constant parallelism in the reactivity of the two antigens. In fact, in many instances, the divergence was marked. For example, the most striking difference was noted in the group of persons with circulating microfilariae. Ninety per cent of this group, irrespective of the degree of microfilaremia, gave positive dermal reactions to the adult-worm antigen, while only 30 per cent reacted to the microfilarial antigen. Other filarial patients tested reacted to one, the other or both of the antigens depending on the degree of infection, the length of exposure, etc. For details of these reactions one should examine the complete report, Franks et al (1946).

Perusal of the data suggests two obvious explanations for the results obtained: (1) that microfilariae provide a reagent different from the adult worm material, not a more specific one, for the diagnosis of filariasis, the microfilarial antigen reacting, as did the antigen of the adult worm, with a homologous antibody in the filarial patient; (2) that the failure of the microfilarial antigen to provoke an intradermal reaction in many of the patients with a microfilaremia was probably due to the presence of circulating microfilariae which fixed or absorbed the antibody that had been produced. In the following experiment it was demonstrated that such antibodies exist and that there is a circulating soluble antigen separable from the microfilariae.

The power to sensitize passively normal persons using sera of filarial patients, Bozicevich and Hutter (1944), who did not have circulating microfilariae, but who reacted to the microfilarial and the adult *D. immitis* antigens, provided a method of demonstrating the presence of at least two distinct antibodies in such sera. Three mixtures were prepared, each containing equal parts of donor serum and one of the following: 1:1000 dilution of microfilarial antigen, 1:8000 dilution of *D. immitis* antigen, or physiological saline solution. The preparations were incubated for two hours at 37° C. and the recipients were given three intradermal injections of 0.1 ml of each of the mixtures. Seventy-two hours later one of each of the three

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[2] The Bureau of Medicine and Surgery of the United States Navy does not necessarily undertake to endorse the views expressed in this paper.

differently sensitized sites was injected intradermally with 0.025 ml of one of the following materials: physiological saline solution, 1:1000 dilution of microfilarial antigen, or 1:8000 dilution of adult-worm antigen. Control tests with the same materials were performed on unsensitized sites on the recipients and were negative. As shown in Table 1, the sensitizing power of the reagin in the mixture had been lost to the homologous antigen.

A similar experiment was set up to attempt to show circulating antigen in the sera of patients with a microfilaremia, who had reacted to neither the microfilarial nor the adult-worm antigens, by substituting their sera, which had been freed of microfilariae, for the antigen in the mixture used to sensitize recipients. The results were inconclusive as there was only a partial loss of the sensitizing power of the reagin in the donor sera.

TABLE 1.—*Intradermal reactions with* D. immitis *microfilarial and adult-worm antigens in recipients sensitized 72 hours previously with reagin-antigen mixtures*

Sensitizing mixture		Recipients tested with					
		H.A.F.			M.B.F.		
Reagin	Absorbed with	Microfilarial antigen 1:1000	Adult antigen 1:8000	Saline control	Microfilarial antigen 1:1000	Adult antigen 1:8000	Saline control
Serum from a filarial patient	(Unabsorbed) equal parts of saline	12×12*	13×11*	3×3	10×13*	10×11	3×4
	Equal parts of 1:1000 *D. immitis* microfilarial antigen	6×6	9×8*	0	4×5	12×12	3×3
	Equal parts of 1:8000 *D. immitis* adult antigen	9×6†	0	2×3	6×8†	0	3×2
Unprepared sites		3×3	4×5	3×3	0	0	0

Measurements in mm.
* Pseudopodia present.
† The *D. immitis* antigen was prepared from adult female worms that contained larvae; this possibly accounts for the partial loss of microfilarial sensitizing reagin.

The presence of a circulating antigen was detected, however, in the following experiments. Several patients, who had varying degrees of microfilaremia and who gave negative dermal responses to the microfilarial and adult-worm antigens, were bled when their circulating microfilariae were at a maximum in the periodicity cycle. The sera were centrifuged to remove all microfilariae and then were employed as follows: 0.025 ml of serum was injected intradermally in non-filarial and filarial patients and the reactions were compared to those elicited by microfilarial and adult-worm antigens. The reactions were of an immediate type and reached their height in 12–15 minutes. Right angle diameters of the wheal were measured and a reaction was considered positive when the wheal caused by the antigen had a diameter twice that of the wheal caused by the physiological saline solution. All the necessary control tests, e.g., normal human serum, dog serum, etc., were performed. The results of a series of skin tests, in which the serum of a patient who had a microfilaremia of 31,000 per ml was employed, are shown in Table 2. Additional evidence of the presence of a circulating soluble antigen was the ability of these sera to give strongly positive intradermal reactions in normal persons who had been sensitized passively with sera from filarial patients who gave strong dermal responses to the microfilarial and adult-worm antigens. In still other experiments several

serum-antigens tested provoked intradermal responses in dilutions of from 1–8 to 1–128.

Although most of the sera employed gave dermal responses in filarial patients, the strongest reactions were noted when the sera of the persons with the higher microfilarial counts were utilized. The question arose as to whether or not the content of soluble antigen of a serum would vary in relation to the periodic rise and fall of the circulating microfilariae. The following experiment was designed for the study of this point.

The sera of two patients, whose peak counts during the cycle were, respectively, 3,400 and 31,000 microfilariae per ml, were bled every three hours for 24 hours. At each bleeding, the degree of microfilaremia was determined by dilution counting (Franks et al, 1946) and serum was collected for use as antigen and thoroughly centrifuged to remove all microfilariae. Each set of sera was tested on three filarial

TABLE 2.—*Intradermal reactions in non-filarial and filarial persons using a serum-antigen from a patient with a high microfilaremia. Reactions are compared with those of other antigens*

Patient	Clinical diagnosis	Serum antigen	*D. immitis* microfilarial antigen 1 : 1000	*D. immitis* adult antigen 1 : 8000	Saline control
T.K.	Filariasis	10 × 11*	12 × 10	15 × 14*	0
A.T.	Filariasis	7 × 8	10 × 10*	11 × 10*	0
T.S.	Filariasis	12 × 13*	16 × 10*	15 × 12	0
T.H.	Filariasis	12 × 12*	10 × 9*	10 × 9*	0
N.S.	Filariasis	9 × 10*	10 × 10*	11 × 7*	0
Y.S.	Filariasis†	7 × 7	0	16 × 11*	0
O.S.	? Filariasis	0	0	7 × 6	0
I.S.	Filariasis	8 × 8	10 × 15	11 × 9	0
N.C.	Filariasis†	6 × 6	0	9 × 9	0
K.T.	Filariasis	10 × 10	11 × 10	11 × 12*	0
K.K.	Filariasis†	6 × 5	8 × 7	13 × 7	0
S.U.	Filariasis†	0	0	9 × 8	0
G.K.	Filariasis†	0	0	6 × 5	0
S.F.	Filariasis†	0	5 × 5	0	0
W.H.	Filariasis	9 × 7*	0	8 × 8	0
I.S.	Filariasis†	7 × 8*	8 × 5	15 × 9*	0
M.B.F.	Normal	0	0	0	0
H.H.F.	Normal	0	2 × 3	4 × 5	3 × 3
J.R.F.	Normal	2 × 3	2 × 3	3 × 3	3 × 3
R.B.T.	Normal	0	0	2 × 3	0
R.G.	Normal	0	0	0	0

Measurements in mm.
* Pseudopodia present.
† Microfilariae found.

patients who were sensitive to both the microfilarial and adult-worm antigens. These patients were injected intradermally with 0.025 ml of the sera drawn at the various times during the filarial periodicity cycle; the results were read 15 minutes after the injections were made. The serum provided by the person with the low microfilarial count gave positive dermal reactions, but there was no difference in the degree of reactivity in the specimens of serum representing the several levels of microfilaremia. However, when the serum of the person with the high microfilarial count was employed as antigen, a difference in the intensity of the dermal responses caused by the specimens of serum obtained during the several bleeding periods was demonstrated in two of three patients receiving the material. In this instance the area differences in the tracings of the reactions were not markedly significant at the different microfilarial levels, but the reactions with pseudopodia were taken to indicate a more intense reaction. A study of the tracings of these reactions suggests that the extent of the reaction may possibly hold an inverse relationship to the degree of microfilaremia present at the time when the specimens were drawn. Conditions beyond control limited a more through investigation of this phenomenon. In Fig. 1 tracings

of intradermal reactions elicited on one of the recipients are plotted in relation to the curve representing the periodicity of the microfilariae in the person supplying the specimens of serum employed in the tests.

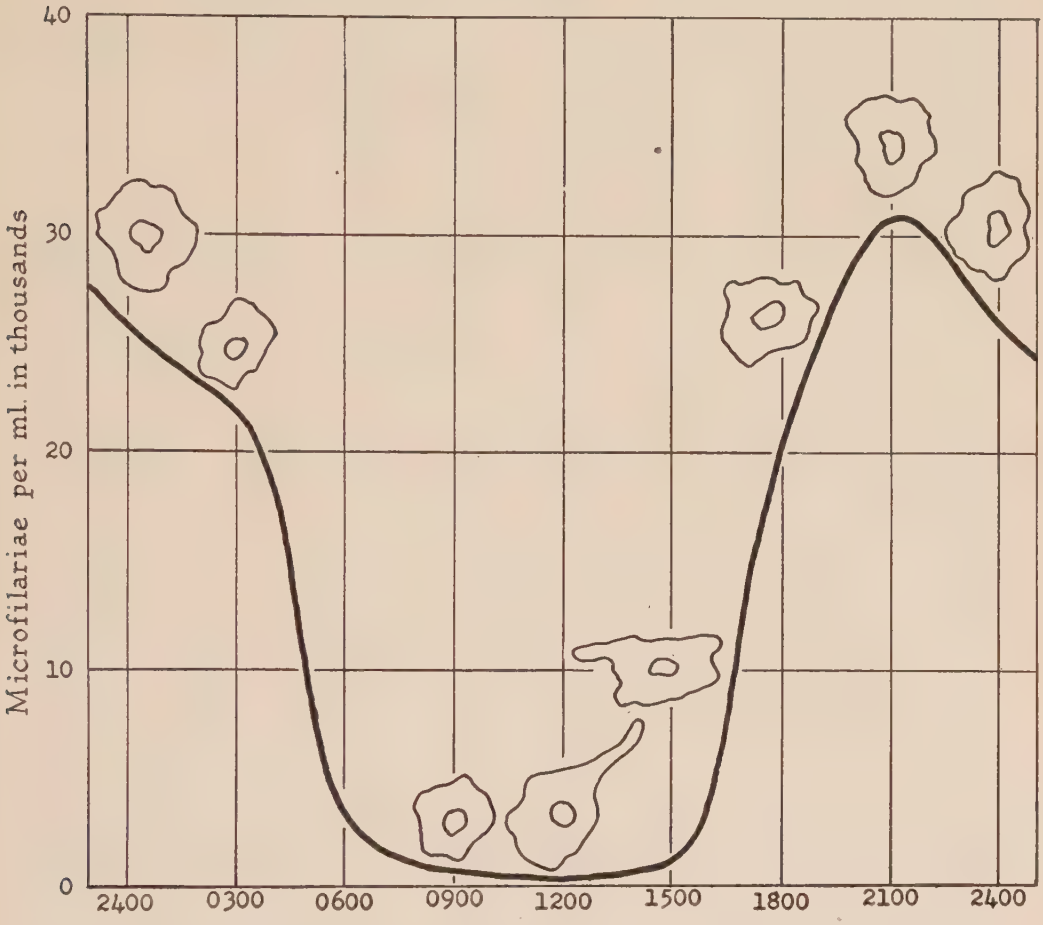

FIG. 1. Tracings reproduced actual size of the intradermal reactions elicited with specimens of serum from a filarial patient. The reactions are plotted in relation to the degree of microfilaremia that was present at the time when the specimens were drawn. The inner circle represents the outline of the initial bleb.

DISCUSSION

The isolation of circulating microfilariae, Franks and Stoll (1945), provides a reagent that is an aid in defining and explaining some of the immunological manifestations of filariasis. Theoretically, in a study of this nature, material prepared from *Wuchereria bancrofti* would be more specific than that from *Dirofilaria immitis*. However, antigens prepared from *D. immitis* have repeatedly given intradermal reactions in human beings infected with filariasis; thus, there is apparently a great deal of similarity between the corresponding antigens of these two helminths. Any small differences in their immunological constitution are probably masked by the group-reacting factor.

The demonstration of circulating antibodies and a soluble antigen explains in

part the variety of dermal responses noted in the infected population studied on Okinawa. It is apparent that the antibody-forming mechanism in the filarial patient recognizes small antigenic differences in the worm material, and that antibodies produced are not directed toward the worm as a whole but against the various antigenic fractions. It has already been shown that antibodies can be produced which are active against isolated tissues of the round worm, *Ascaris lumbricoides* var. *suum,* Oliver-Gonzales (1943); with suitable antigenic material, antibodies specific for the coelomic fluid, intestine and other fractions of the filarids could probably be detected.

It is clear from the experiments demonstrating the presence of a circulating soluble antigen, that the absence of antibodies in many of the patients, who unquestionably were infected with filariasis (i.e., those with a microfilaremia), can be accounted for by their continuous removal through combination with an excess of the soluble antigen constantly present. It is possible that there is more than one soluble antigen in the circulation related to the microfilariae; in addition, there may be soluble antigens related to the adult worm, since it was shown that the serum-antigen reacted in patients who were sensitive to the adult worm but not to the microfilarial antigen. In other instances, sera provided by filarial patients without circulating microfilariae gave strong dermal reactions. It is interesting to note that these antigens resemble toxins in certain ways. A toxin-like material from the worm is believed to precipitate attacks of filarial fever, Acton and Rao (1930), and to cause lesions in lymph nodes and lymph channels that the worms themselves have not invaded, Michael (1945). It has been noted, too, that even the small amounts of antigen employed in the intradermal injections for diagnosis have been followed by exacerbations of lymphangitis and pain in the filarial patients, Bozicevich and Hutter (1944).

It is, of course, hazardous to relate the amount of antigen in a serum to the intensity of the reaction provoked when that serum is injected intradermally. The occurrence of a tide of antigen in a person whose circulation is continuously flooded with microfilariae is entirely plausible; however, the results of these experiments can be accepted only as tentative evidence of such a phenomenon until more data are accumulated. Theoretically at least, the person providing the specimens of serum for these experiments had a considerable quantity of antigenic material in her circulating blood. As computed on the basis of dry weight of the total number of microfilariae present in all the circulating blood,[3] the weight of the microfilariae in circulation varied from 5 mgm at the low tide of the filaremia to 1000 mgm at the peak of the cycle.

It is of interest to note that in these experiments the tide of antigen was more easily detected when certain optimal conditions were satisfied in the choice of sera for testing and in the selection of the recipients. The small differences in the content of antigen of the sera were most consistently or easily demonstrated when the specimens of serum employed were provided by a person with a high microfilarial count. The reason why such sera give the best results may reside in the fact that there is

[3] Calculations of several specimens of microfilariae of *D. immitis,* isolated from the blood, indicated that there were 400–500 million in a gram, dry weight, Franks et al (1946). Computed on a basis of comparable size of the microfilariae of *W. bancrofti* and *D. immitis,* a gram, dry weight, would contain about 200 million *W. bancrofti* microfilariae.

a large rise and fall in the number of circulating microfilariae in the periodicity cycle. The differences, too, were more easily detected when the specimens of serum were tested on persons who gave the strongest dermal reactions to the microfilarial and adult-worm antigens.

Several attractive hypotheses might be offered to explain the stronger dermal responses (i.e., greater concentration of antigen) at the low ebb of the microfilarial tide. The simplest explanation is that the antigen and the microfilariae are released into the circulation at the same time and in the lag of from 10 to 12 hours, which occurs while the microfilariae are reaching their peak level, the concentration of antigen decreased considerably. This theory would hold regardless of whether the microfilariae migrate from the lungs and other tissues into the circulation or make their appearance as the result of the daily cyclical parturition of larvae. The concentration of antigen also may be directly related to the microfilaremia, with a lag occurring in the appearance of the antigen in the circulation. Moreover, there may be a tide of antibody whose gradual rise parallels that of the microfilariae, so that what is measured is the excess of antigen not in combination with antibody. Possibly, in the delicate balance of an antigen-antibody system lies the explanation of the periodicity of the microfilariae.

These experiments are of a practical as well as a theoretical interest. If serological and skin tests are necessary for confirming a diagnosis of filariasis, such tests should be set up within the limitations of the immunological principles involved. Apparently, no one antigen is entirely suitable for confirmatory tests in individual cases of filariasis. Moreover, it is reasonable to assume that a series of tests in which are measured the different antibodies and antigens in the circulating blood would increase the chances of making definite diagnoses. An antigen prepared from the adult female worm cannot be considered a suitable polyvalent antigen because of the quantitative disproportion of its several antigenic fractions of cuticle, larvae, coelomic fluid, etc. Some of these fractions may be present in such small amounts that they will not react at all, and may even be completely lost or denatured in the preparation of the antigen. The experiments demonstrating a soluble antigen in the blood suggest that the sera of filarial patients or of dogs infected with *D. immitis* might provide suitable antigens to be used in the diagnosis of filariasis. An antigen prepared from the serum of a person or of a dog infected with filaria would have many advantages. It would be free of many of the inert substances and irritating radicles that are present in preparations made from the whole worm. Furthermore, the assay of the material would be relatively simple as it could be tested on filarial patients or against hyperimmune sera prepared from isolated fractions of the filarid. In addition, the antigen could be concentrated easily, and it is possible that specific fractions could be isolated. Finally, large amounts of material would be easier to obtain from such a source than from the adult worm or microfilariae.

SUMMARY

Two antibodies have been demonstrated; one specifically directed against the microfilariae, the other against the adult filarid. The presence of circulating soluble antigen was detected in the sera of a large number of persons who had a microfilaremia and also in the sera of a few filarial patients who did not have circulating microfilariae. Preliminary experiments indicate that sera containing this antigen

(or antigens) might be utilized for diagnostic tests in filariasis. In one experiment, the results suggest that this antigen (or antigens) appears in the blood as a tide whose ebb and flow are related inversely to the different concentrations of the microfilariae in the circulation.

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A NEW SPECIES OF *RHOPALOPSYLLUS* BAKER (SIPHONAPTERA) FROM TEXAS

RICHARD B. EADS

Division of Entomology, Bureau of Laboratories, Texas State Department of Health, Austin, Texas

In connection with a murine typhus research program being conducted by the Texas State Department of Health in cooperation with the United States Public Health Service in Lavaca County, Texas, a large number of domestic rats and wild animals are being combed for ectoparasites. A good series of an apparently new *Rhopalopsyllus* flea have been taken from raccoons, opossums, a Texas red wolf, and an armadillo. Dr. C. F. W. Muesebeck kindly checked for the presence of this species in the United States National Museum. It is described in this paper and is being named for Dr. George W. Cox, Texas State Health Officer.

Family DOLICHOPSYLLIDAE

Rhopalopsyllus (*Rhopalopsyllus*) *coxi* n. sp.

Holotype Female

Head: Genal and pronotal ctenidium absent; frontal tubercle prominent and dorsally directed; antennae large; a row of very small, stout setae along the margin of the antennal groove; preantennal region of the head with two rows of bristles, the upper row consisting of five bristles with the one nearest the antennae long and stout and the other four considerably weaker, the lower row is composed of three large, stout bristles; a large bristle present on gena just back of the eye, and another below the eye; the labial palpi extend beyond the fore coxae; postantennal region of the head with three rows of bristles.

Thoracic and Abdominal Segments: Prosternum extending downward anteriorly between the coxae; mesosternum as high as long; pro- and mesonotum with two rows of bristles, the metanotum with three; each abdominal tergite with a row of stout bristles and a second less regular row of delicate bristles; opening of spiracles large; one pair of antepygidial bristles present; sternite X normal for the genus; sternite VII rounded, without a sinus, and with five delicate bristles in a row; receptaculum seminis long and slender, head almost four times as long as wide, swollen at base in shape of a club, and longer than tail.

Legs: Fifth tarsal segment armed with three pairs of stout, lateral, plantar bristles, a fourth pair of smaller ones, and an apical submedian pair.

Allotype Male

Head and thorax: General structure and chaetotaxy similar to female.

Modified segments: Clasper large, the dorsal margin with a number of large bristles, and with two large, heavily pigmented bristles and a more delicate one terminally; movable finger large, with fine setae on the margins, curved against clasper but not reaching apex of clasper; posterior arm of sternite IX pointed apically and bearing numerous bristles.

Holotype: A female taken from a raccoon, *Procyon lotor,* from Borcher's Ranch, Hallettsville, Texas, by G. H. Halyard, January 9, 1946. Retained in the collection of the Texas State Health Department.

Allotype: A male, collected and deposited as holotype.

Paratypes: The following collections have been made from Hallettsville, Texas: Two specimens from opossum, *Didelphis* sp., November 14, 1945, collecter E. G. Batte; three specimens from raccoon, *Procyon lotor,* December 13, 1945, collector Halyard; five specimens from opossum, *Didelphis* sp., December 19, 1945, collector Batte; two specimens from opossum, *Didelphis* sp., December 20, 1945, collector Halyard; one specimen from Texas red wolf, *Canis rufus,* January 7, 1946, collector Batte; two specimens from raccoon, *Procyon lator,* January 9, 1946, collector Batte; two specimens from raccoon, *Procyon lotor,* January 10, 1946, collector

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Halyard; one specimen from armadillo, *Dasypus novemcinctus,* December 6, 1945, collector Halyard; one specimen from raccoon, *Procyon lotor,* January 10, 1946, collector Halyard.

Type host: Procyon lotor.

Type locality: Hallettsville, Texas.

Remarks:—This new species appears to be a typical member of the subgenus *Rhopalopsyllus.* The only other species of the genus *Rhopalopsyllus, R. gwyni* and

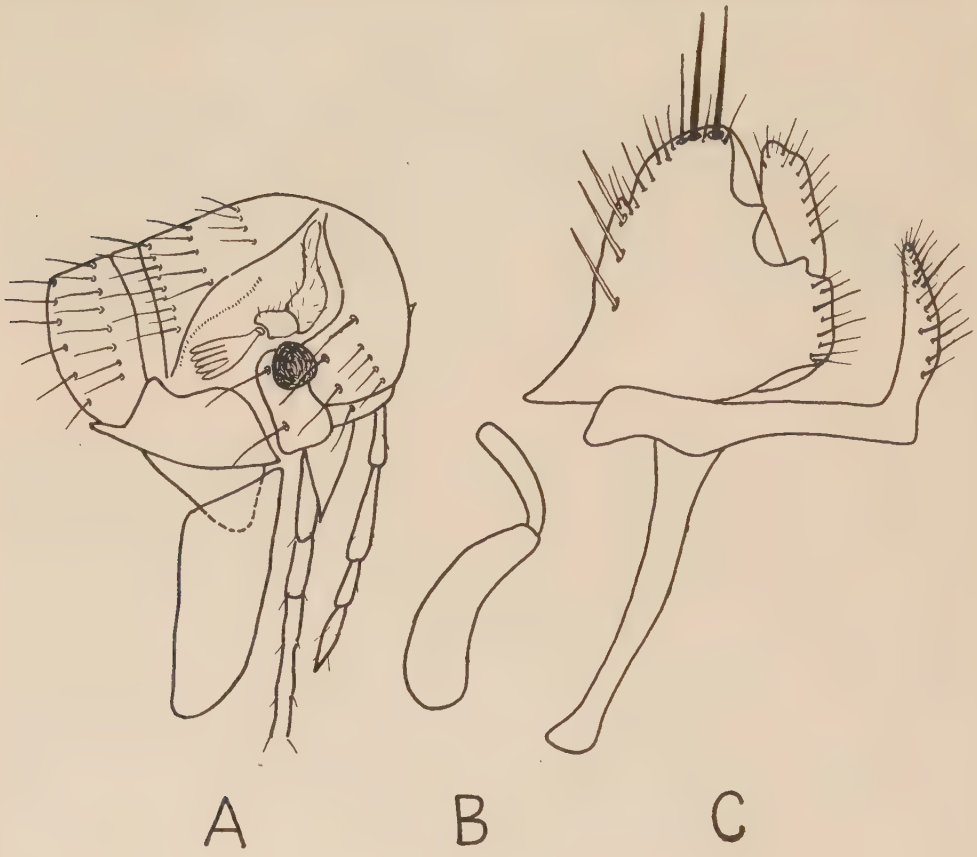

PLATE I

A. Head of *Rhopalopsyllus coxi,* n. sp., holotype female.
B. Receptaculum seminis of *R. coxi,* n. sp., holotype female.
C. Genitalia of *R. coxi,* n. sp., allotype male.

R. sigmodoni, recorded from the United States belong to the subgenus *Polygenis.* In the subgenus *Rhopalopsyllus* the prosternum extends downward anteriorly between the coxae, and the mesosternum is as high as long. In the subgenus *Polygenis* the mesosternum is not as high as long, and the prosternum does not extend downward between the coxae. These two characters readily separate *R. coxi* from *R. gwyni* and *R. sigmodoni.*

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THE ENDOGENOUS PHASE OF THE LIFE CYCLE OF *EIMERIA BOVIS*[1]

DATUS M. HAMMOND,[2] GEORGE W. BOWMAN, LEONARD REID DAVIS, AND B. T. SIMMS

INTRODUCTION

During the past several years an intensive study of bovine coccidia has been under way at this laboratory. Since *Eimeria bovis* is so widespread and causes such serious losses, it has received more attention than the other species. Christensen (1941) pointed out that *E. smithi* is a synonym of *E. bovis,* redescribed the unsporulated oöcyst, and determined the time required for sporulation under optimum conditions. Boughton (1942) reported that the cyst-like structures in the mucosa of the small intestines of cattle described under the names of *Globidium* and *Gastrocystis* are the schizonts of *E. bovis.* He (1943) found also that sulfaguanidine can be used as a preventive in experimentally inoculated calves. Hammond, Davis, and Bowman (1944) described the symptoms and pathology observed in calves inoculated with this species, and recorded a definite relation between the size of the inoculum and the symptoms produced.

This paper reports the results of further studies of this parasite with emphasis on the endogenous phase of its life cycle.

MATERIALS AND METHODS

Fifty-one calves were used in these experiments. They were procured and placed in individual pens when not more than 2 days old. The method of feeding and caring for them has been described elsewhere (Hammond, Davis, and Bowman, 1944). A majority of the calves had infections, usually light, with other species of coccidia before they were inoculated with *E. bovis.* Some had very light infections with *E. bovis* prior to inoculation.

The oöcysts used in these studies were obtained from the feces of both naturally and artificially infected calves. Sporulation was induced by the technic already described (Hammond and Davis, 1944). Sporulated oöcysts were placed in 2 per cent potassium bichromate solution and stored in a refrigerator. Before they were used the potassium bichromate was removed by centrifugation and decanting.

Schizonts and merozoites of *E. bovis* were obtained from scrapings of the intestinal mucosa of infected calves. They were separated from intestinal debris by light centrifugation. Immediately before use they were checked for viability by crushing some of the schizonts under a cover slip and examining the liberated merozoites for motility.

Intestinal fluid was prepared by passing the liquid portion of the intestinal content of calves through filter paper. The fluid in the anterior half of the small intestine was collected separately from that in the posterior half.

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[1] From the U. S. Regional Animal Disease Research Laboratory, Bureau of Animal Industry, Agricultural Research Administration, U. S. Department of Agriculture, Auburn, Alabama.

[2] Resigned December 15, 1944.

Two of the 51 calves received inoculations directly into the intestine. In one of these a laparotomy[3] was performed when the calf was 1 week old, and viable schizonts and merozoites were injected directly into the ileum with a hypodermic syringe. Following this, fecal examinations were made daily to determine when oöcysts appeared and disappeared from the excrement. In the second of these calves a Thiry's fistula was established when the calf was about 4 months old. This involved a segment of the ileum about 1 foot long and located about 6 feet anterior to the ileocecal valve. This calf was used in two types of experiments. One, performed 4 times, consisted in introducing sporulated oöcysts, enclosed in cellophane bags, into the isolated segment of the intestine, leaving them there for 18 to 46 hours, removing the bags, and examining the oöcysts for evidence of excystation. The other, performed twice, consisted in introducing viable schizonts and merozoites directly into the segment and making daily examinations of curetted mucosa from this segment for evidence of oöcyst formation.

Forty-nine of the 51 calves were inoculated *per os* with large numbers (1 to 20 millions) of sporulated oöcysts of *E. bovis* (Plate I, Fig. 1) following the technic previously described (Hammond, Davis, and Bowman, 1944). Thirty-nine of these inoculated calves were held under observation without any treatment. To obtain information about excystation, 7 of the 39 were killed at the 15th to the 46th hour following inoculation. The contents of each 6-foot section of the small intestine and of the cecum and colon were removed from these animals as quickly as possible and kept warm while being examined for oöcysts and sporozoites. The other 32 of these calves were killed on the 2nd to the 30th day following inoculation to obtain information about schizont and sexual stages. Most of those destroyed after the 20th day had developed acute symptoms of coccidiosis, 10 being moribund when they were destroyed.

In 6 of these 49 calves attempts were made, at the time of inoculation, to produce partial stenosis of the intestine by surgical means. The site of the attempted stricture was along the ileum about 8 to 12 feet anterior to the ileocecal valve. It was hoped that this stricture would retard movement of ingesta along the digestive tube and result in concentration of the developing asexual stages of coccidia in the mucosa just anterior to this site. These 6 calves were destroyed on the 4th to the 14th day following their operations.

Four of the 49 calves were subjected to enterectomies on the 16th to the 18th day following inoculation. In 3 of these a portion of the blind end of the cecum as well as a segment of the ileum was excised, while in the fourth, only a segment of the ileum was removed. Excised segments were examined for schizonts, fixed, sectioned, and stained. One of these animals showed alarming symptoms almost immediately after the operation and was destroyed when moribund 24 hours later. The other 3 developed typical symptoms of acute coccidiosis and were destroyed on the 5th, 6th, and 8th days, respectively, following the operations.

All of these 49 calves were subjected to postmortem examinations within a few minutes after they were destroyed. At each autopsy about 20 segments, each about one-half inch long, were excised at approximately equidistant points along the entire intestinal tract. These were inverted over sections of tongue depressors to prevent

[3] Dr. A. B. Larsen, of this laboratory, assisted with all the surgical operations which were performed on calves used in these studies.

curling and placed in fixing fluid. Zenker's fluid was used regularly as a fixative; occasionally alternate or other segments were fixed in Helly's fluid, Schaudinn's fluid, Bouin's fluid, or formalin. Sections were routinely stained with iron-haematoxylin, haematoxylin and eosin, and Giemsa. In some cases Goldhorn's polychrome methylene blue and eosin, as recommended by Becker and Roudabush (1939), Heidenhain's azan, and Kornhauser's quadruple stains, were used. At each autopsy scrapings of mucosa from the different parts of the intestine were examined for coccidia. If schizonts containing merozoites were found, smears were made and stained with Wright-Giemsa or Giemsa stain.

Four attempts to produce excystation of oöcysts *in vitro* by incubating them at 37° or 40° C for 18 to 24 hours in intestinal fluid of calves were made. Repeated attempts to produce excystation by following the technic employed by Goodrich (1944) in working with *E. tenella* and *E. stiedae* were made.

EXCYSTATION

Sporozoites were found in 5 of the 7 calves killed 15 to 46 hours after inoculation. In the 3 calves killed 15 to 18 hours after inoculation the sporozoites were present in the contents of the small intestine taken from the region between 6 and 16 feet anterior to the ileocecal valve. A single sporozoite was seen in scrapings from the mucosa about 9 feet anterior to the ileocecal valve in each of the 2 calves killed 30 and 46 hours, respectively, after inoculation. In the 2 calves killed 21 and 24 hours after inoculation, respectively, no sporozoites were found in the contents of the intestine, but in these 2 cases a careful examination of scrapings from the mucosa was not made.

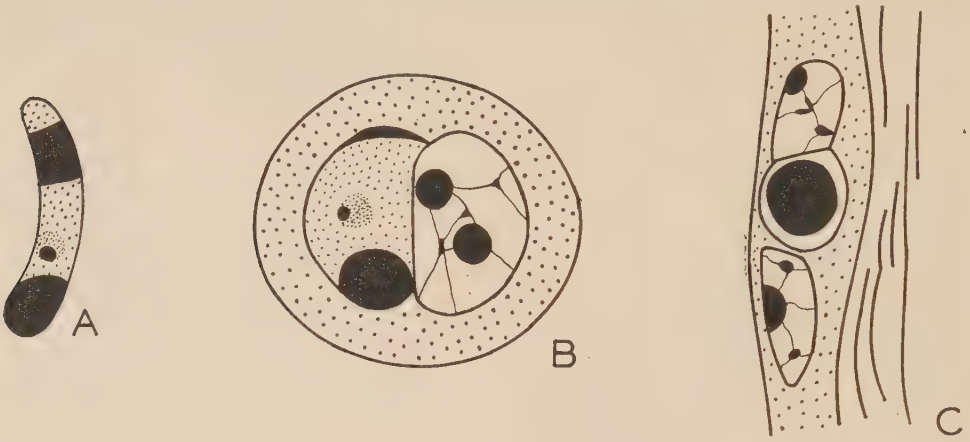
In the 3 calves killed 15 to 18 hours after inoculation oöcysts in various stages of excystation were found in the posterior part of the small intestine, cecum, and colon. In many oöcysts the outer wall was incomplete at the narrow end and this area was indented in a number of cases. Some oöcysts had openings at the narrow end; in some a few of the sporozoites had escaped from the oöcyst, while in others all of the sporozoites had escaped, leaving only the sporocysts, empty except for residual matter. In some oöcysts with no perceptible opening in the wall, some of the sporozoites were free of the sporocyst. Also, many wrinkled oöcysts with intact contents were seen. In the calf killed 21 hours after inoculation no oöcysts were found in the small intestine, and only a few were present in the cecum and colon; some of these had partially or completely excysted. In the calves killed 24 to 46 hours after inoculation no oöcysts were found on direct examination of material from any part of the intestine, but a few unsporulated and degenerate oöcysts were found on flotations of material from the cecum and colon.

These findings indicate that under the conditions of these experiments most of the sporulated oöcysts in an inoculum passed through the intestine within 24 hours, and that only a small number completed excystation. The sporozoites were apparently set free in the posterior part of the small intestine, beginning not later than 15 hours after ingestion of the oöcysts.

The only sporozoite showing motility was found in a calf killed 46 hours after inoculation. This sporozoite moved in a counter-clockwise spiral path, the anterior half of the body being flexed occasionally from one side to another. It continued moving at a progressively slower rate for the period of approximately 1 hour that it was under observation.

The sporozoites were 10.5 to 13.5 microns long and 3 to 4 microns wide. The posterior end was more bluntly rounded than the anterior, and the body was usually slightly curved. They appeared light-green, yellow, or yellow-green. There was a relatively large refractile body in the posterior end, and a slightly smaller one in the anterior end, with a lighter area between the two.

In sections of sporulated oöcysts stained with iron-haematoxylin, sporozoites were found to contain two deeply staining bodies similar in size and position to the refractile bodies seen in the living sporozoite (Text figure 1, A). The relatively small nucleus with its eccentric karyosome was located in the space between the two deeply staining bodies. Sporozoites were not identified in sections of intestine.



TEXT FIGURE 1. The sporozoite and early schizont stages, sketched with the aid of a camera lucida from sections stained with iron-haematoxylin. A. Sporozoite, with nucleus near center. B. Early schizont in host cell whose nucleus is at right, with crescent-shaped body of schizont at top, nucleus near center, and spherical body at bottom. C. Schizont in endothelial cell lining central lacteal, with only spherical body of schizont showing (remaining portion in adjacent section). ×2000.

When oöcysts in a closed cellophane bag were left 18 to 46 hours in the Thiry's fistula the sporocysts were liberated, but the sporozoites remained within the sporocysts. In each of the four trials numerous free sporocysts were seen (Plate I, Fig. 2), but no free sporozoites. The segment must have contained enzymes of the intestinal glands only, and apparently some agents or conditions necessary to bring about complete excystation were lacking.

In two of four experiments some of the oöcysts incubated for 18 hours at 40° C in filtered fluid from calves' intestines showed evidence of excystation similar to that found in the experiments in calves, but no free sporozoites were seen. In one of the two experiments the fluid from the anterior portion and that from the posterior portions of the small intestine were incubated separately; it was in the anterior portion that the excysted oöcysts were seen. In the other experiment one portion of the fluid was incubated at 37.5° C, while another portion was incubated at 40°; no difference in results in these two portions was noticed. In this experiment oöcysts were also found which were completely empty, in contrast to the excysted oöcysts mentioned above, which still contained empty sporocysts.

In a third experiment, in which the fluid was incubated for 24 hours at 40° C, there were some oöcysts from which some or all of the sporocysts had escaped; some

free sporocysts were seen, but no free sporozoites were observed. In the fourth experiment, conducted in a similar way, the only alteration observed was an indentation at the narrow end in some of the oöcysts.

In all of these experiments there was rapid multiplication of bacteria during the period of incubation, and this may have been a limiting factor in excystation of the oöcysts.

The results of these experiments and those with the Thiry's fistula indicate that under certain artificial conditions the intact sporocysts may escape from the oöcyst, but the results of the observations in calves indicate that under natural conditions the sporozoites are set free within the oöcyst and escape, leaving the sporocysts with only residual material.

Goodrich (1944) has described a procedure whereby she was able to observe excystation of oöcysts of *E. tenella* and *E. stiedae* under the microscope. Repeated attempts to obtain similar results with this method on the oöcysts of *E. bovis* were unsuccessful. A number of free sporocysts and a few sporozoites were seen, but these were probably liberated through the action of the mechanical forces employed.

ASEXUAL STAGES

Location

The schizont stage of *E. bovis* is unusual among the coccidia in that the parasite, in this stage, attains sufficient size to be detected in the intestine with the unaided eye. Schizonts are located in the center of villi, appearing as white, opaque bodies. Since all but younger schizonts are macroscopic, their occurrence and distribution in an infected intestine can be readily determined.

In most cases schizonts were present only in the posterior half of the small intestine. Rarely, schizonts were also found in the cecum and upper colon. Usually the maximum concentration occurred about 10 feet anterior to the ileocecal valve. In calves killed at the height of development of schizonts (16–18 days after inoculation) the maximum concentration of schizonts large enough to be seen with the unaided eye ranged between 25 and 400 per sq. cm; in several calves it was about 100.

The schizont occurs in a host cell, whose cytoplasm becomes stretched into a thin membrane as development of the parasite progresses. The nucleus of the host cell, with large deeply staining granules, is present all through development (Plate I, Figs. 6 and 9). Outside the host cell is another membrane formed of flattened cells resembling those of endothelium (Plate II, Fig. 14). The whole structure lies in the central lacteal, usually toward the tip. In heavy infections two or more schizonts frequently occur in a single villus (Plate I, Fig. 8 and Plate II, Fig. 10). In cases of this kind the schizonts may be at similar or different stages of development. In one case a schizont was seen in sectioned material in the submucosa under the muscularis mucosae.

The origin and identity of the host cell cannot be stated with certainty. The schizonts, inside their host cells, including the earliest stages found, were invariably located inside the central lacteal, more or less imbedded in its wall, or at the tip of the lacteal. In a number of cases early schizonts were seen in endothelial cells in the endothelial lining of the lacteal[4] (Text figure 1, C). In these cases endothelial cells

[4] This identification has been confirmed by Drs. W. H. Taliaferro and William Bloom of the University of Chicago. Dr. H. R. Seibold of the Pathological Division, Bureau of Animal Industry, assisted in the identification of tissue cells. The authors express appreciation to these men.

were acting as host cells for the schizont, but the possibility that other unidentified cells of the reticular connective tissue may be parasitized cannot be excluded. Frequently, early schizonts were found bulging out from the wall of the lacteal into its lumen, with a cell attached to one or both ends of the ellipsoidal host cell (Plate I, Fig. 5). It seems likely that the adhering cells were those originally lying next to the parasitized cell in the endothelial lining, and that cells of this type are the ones which later form a complete membrane around the host cell.

Early Schizonts

Although a number of calves were killed 2, 3, and 4 days after inoculation, no organisms in any stage were found in fresh material or sections. A few schizonts were found in a calf killed 5 days after inoculation, and they were present in some calves killed 6 days after inoculation. The schizont was situated close to the nucleus of the host cell. The nucleus of the schizont had a deeply staining body, considered to be a karyosome, which was located at the side of a spherical or elliptical area taking a uniform light stain with iron-haematoxylin (Text figure 1, B). This area was separated by a clear space from the cytoplasm. There was no distinct nuclear membrane. In addition to the nucleus, each schizont contained two bodies which stained intensely with iron-haematoxylin, but were eosinophilic with Giemsa stain (Plate I, Figs. 3, 4, and 6). The smaller of these bodies was crescent-shaped, while the larger was spherical. The relative positions of the two bodies varied, but sometimes they were at opposite ends of the schizont with the nucleus in an intermediate position (Plate I, Fig. 3 and Text figure 1, B). Schizonts with this arrangement showed a striking resemblance to sporozoites as seen in sections of oöcysts (Text figure 1, A), the chief differences being in the shape of the body and in the shapes of the two deeply staining structures. The nuclear structure was similar, and in both the schizont and sporozoite the two extra-nuclear bodies stained deeply with haematoxylin, and were eosinophilic (schizonts stained with Giemsa, sporozoites with Goldhorn's polychrome methylene blue and eosin). Tyzzer (1929) found an eosinophilic disc or globule, which stained black with iron-haematoxylin, in the first generation schizont of *E. tenella*. The resemblance of the early schizont to the sporozoite, together with the apparent absence of any intermediate stages, indicates that the sporozoite gives rise directly to the schizont.

Development of Schizonts

Division of the nucleus begins early, since many of the small schizonts in the calf killed 6 days after inoculation showed two or more nuclei. Division occurs by elongation of the karyosome and separation into two parts, sometimes equal in size and sometimes unequal. Thus, the process of nuclear division as well as the structure of the nucleus closely resembles that described for *E. caviae* by Lapage (1940).

In the course of development the spherical deeply staining body breaks up into two or more smaller bodies and these finally disappear, thus indicating that the spherical body is made up of some kind of reserve material used in growth. The crescent-shaped body is extruded into the space between the schizont and the cytoplasm of the host cell (Plate I, Fig. 7 and Plate II, Fig. 10). After the schizont reaches about the half-way stage in its development this body is no longer visible; evidently it undergoes disintegration. Its significance is unknown.

In early stages the nuclei are scattered at random through the schizont, but in those of approximately one-half to two-thirds mature size the protoplasm is arranged in numerous spheres with the nuclei at the surface of the spheres (Plate I, Fig. 9 and Plate II, Fig. 11). In some schizonts studied in fresh preparations the spheres had smooth surfaces. In others, small, evenly spaced protuberances were present on the spheres, and some schizonts were seen in which the spheres had radially arranged projections resembling the tips of merozoites. Some spheres were very small, having only two to four projections, while others were much larger, with a hundred or more projections. These projections were probably stages in the development of merozoites.

The rate of growth and the time that maturity is reached are indicated in Table 1. In the calves killed from the 6th to the 17th day after inoculation the schizonts were progressively larger. The first mature schizonts (with merozoites present) were observed on the 14th day after inoculation. Thus, the time interval between the earliest schizont observed and the appearance of the first mature schizonts was 8 days. A few relatively small schizonts were noted as late as 20 days after inoculation. There was no evidence found to indicate that more than a single generation of schizonts occurs.

Mature Schizonts and Merozoites

Mature schizonts were elliptical, ovoid, or, less frequently, approximately spherical in shape. One hundred mature schizonts measured in fresh preparations without a cover slip averaged 281 by 203 microns. The maximum size was 435 microns long by 265 microns wide; the minimum length was 207 microns, and minimum width, 134 microns. In some schizonts, which were considered not to be fully mature, the merozoites were arranged in more or less regular rows (Plate II, Fig. 13). The pattern formed by these rows was absent in fully mature schizonts, the merozoites being crowded together with no particular arrangement (Plate II, Figs. 15 and 16). When schizonts were crushed under the microscope large numbers of merozoites were liberated. In order to determine the approximate number of merozoites it contained, each of 20 mature schizonts was crushed in 0.2 cc of physiological saline solution, the merozoites were scattered through the solution by stirring, and samples were removed for counting in a hemacytometer. In these schizonts, whose average size was 313 by 198 microns, the average estimated number of merozoites per schizont was 120,000, and the range was 55,000 to 170,000.

Schizonts visible to the unaided eye appeared in greatest numbers in calves killed 16 to 18 days after inoculation. That mature schizonts decreased in number after this time, is indicated by the finding of relatively smaller numbers in the calves killed at 20 days and later, and also by the results in a calf (No. 499 in the table) on which an enterectomy was performed. When this calf was killed on the 23rd day after inoculation, mature schizonts were only about one-third as numerous in the area of the enterectomy as at the time of the operation on the 17th day after inoculation. This reduction in number was probably the result of rupture of mature schizonts and liberation of their merozoites. The mechanism of this rupture is unknown, but in a number of cases the part of the epithelium and that of lamina propria covering the outer portion of the schizont were thinned out in varying degrees, and in one case there was a small gap over the schizont at the tip of the villus.

How long schizonts may remain in the intestine is unknown, but a few were still present in a calf killed 30 days after inoculation. In this case the majority appeared degenerate upon microscopic examination. Fine granules, which underwent Brownian movement, escaped when these schizonts were crushed. In some cases no merozoites were present; in others there were relatively small numbers of non-motile merozoites containing large vacuoles.

Little or no inflammatory reaction of the small intestine was noted on gross examination, even where schizonts were most abundant. In some cases large numbers of eosinophil leucocytes were noted in sections of tissue in which schizonts were present. Otherwise, no pathological change was observed in sections.

Beginning as early as 16 days after inoculation some schizonts were invaded by granulocytes and large mononuclear cells (Plate II, Fig. 14). Schizonts so invaded were usually located away from the tips of villi, toward the base of the mucosa. Various stages of invasion were seen. This phenomenon was more evident in calves with severe infections than in calves with light infections.

TABLE 1.—*Size and time of maturity of schizonts relative to time after inoculation*

Calf No.	Days after inoculation	Size (in microns) of 50 schizonts			Per cent mature
		Average size	Maximum size	Minimum size	
599	6	8.6 × 5.9	16.1 × 11.2	7.7 × 4.9	0
557	7	18.0 × 14.4	30.8 × 23.1	7.7 × 4.9	0
528	8 and 9*	43.9 × 30.3	78.3 × 49.3	9.1 × 5.6	0
487	10	54.5 × 43.5	101.5 × 81.2	11.5 × 10.0	0
523	11 and 12*	83.3 × 51.9	217.5 × 98.6	9.1 × 5.6	0
514	12	88.1 × 53.1	171.1 × 72.5	23.2 × 20.3	0
576	14	130.0 × 86.7	230.4 × 140.8	12.6 × 11.2	12
499	17	180.5 × 114.7	275.2 × 160.0	30.0 × 30.0	60
591	20	157.9 × 108.1	256.0 × 128.0	15.0 × 15.0	60
499	23	161.5 × 110.9	288.0 × 108.8	64.0 × 51.2	70
589†	27	266.4 × 182.1	384.4 × 160.0	185.6 × 134.4	90
597†	30	327.7 × 230.0	435.2 × 236.8	243.2 × 198.2	100‡

* Two inoculations, second about 16 hours after first.
† Observations made on fresh material.
‡ Many schizonts appeared to be degenerate.

The merozoites usually showed motility in physiological saline solution for a short while after liberation from the schizont. Two types of movement were seen; in one, the anterior end of the body was slowly flexed back until it touched the middle, then quickly straightened. In the other, the merozoites executed gliding movements, usually in a curved pathway, but occasionally in a spiral. The body was rounded at the anterior end, and abruptly tapered to a point at the posterior end. Specimens examined in fresh preparations showed no internal structure, but in those stained with Wright-Giemsa a nucleus was visible near the posterior end and a variable number of granules was observed anterior to it (Plate II, Fig. 12). Two hundred merozoites measured in fresh preparations ranged between 8.8 and 15.4 microns in length, the average being 11.6; most were about 2 microns in width. Occasionally schizonts were observed which contained longer and more slender merozoites than usual but no sexual dimorphism was noticed.

Relationship Between Schizonts and the Sexual Stages

In both of two trials, oöcysts of *E. bovis* were recovered following introduction, into the Thiry's fistula, of schizonts and merozoites obtained from calves 23 and 17 days, respectively, after inoculation of oöcysts. In the first trial oöcysts were found

only on the 5th day following introduction of schizonts and merozoites, while in the second trial oöcysts were recovered from the 4th to the 16th day.

Injection of schizonts and merozoites, obtained from a calf killed 17 days after inoculation of oöcysts into the ileum of a week-old calf, was followed by discharge of *E. bovis* oöcysts from the 5th to 15th day following the injection. The concentration of oöcysts was highest on the 5th day, and on this and the following day there was a slight bloody diarrhea. Oöcysts of *E. ellipsoidalis* were present in the fecal samples on the 4th, 9th to 11th, and 14th to 16th days. It is possible that these oöcysts originated from the injection, since an immature *E. ellipsoidalis* oöcyst was found in the small intestine of the calf from which the schizonts and merozoites were obtained.

In a calf in which stenosis of the ileum was brought about surgically just prior to inoculation with oöcysts, the concentration of schizonts was heaviest in the region from about 3 feet anterior to the site of stenosis to about 2 inches posterior to it. This distribution may be explained on the assumption that the schizonts originate either directly or indirectly from the ingested oöcysts.

Sexual Stages

Parasites in the sexual stage were usually found only in the cecum and colon. However, in heavy infections such parasites were frequently observed in progressively diminishing numbers from the ileocecal valve to 3 or 4 feet anterior to the valve in the ileum. In the three calves which lived for 5 or 6 days after enterectomy, oöcysts were found in scrapings from the mucosa in regions much farther forward in the small intestine than usual, in one case extending within 12 feet of the pyloric valve. In these cases the anastomosis had apparently caused a partial obstruction in the small intestine, as evidenced by the presence of higher concentrations of parasites in oöcysts in sections of the area just anterior to the point of anastomosis than in sections of the area just posterior to it.

Parasites in the sexual stage were found in the cytoplasm of the epithelial cells of the intestinal glands. Usually the cells at the base of the gland were attacked first, and later the rest of the gland became involved. In some heavy infections a few oöcysts were also seen in the submucosa.

Parasites in the earliest sexual stages found were in tissues from a calf killed 17 days after inoculation; however, it seems certain that such stages occur earlier than this, because discharge of oöcysts occasionally began on the 18th day. In the early stages sexual differentiation could not yet be distinguished (Plate III, Fig. 17). In later stages macrogametocytes were identified by the presence of granules in the cytoplasm, and by a relatively large nucleus with prominent karyosome, while microgametocytes possessed several nuclei (Plate III, Fig. 18). Macrogametocytes and microgametocytes were present in the same region of parasitized glands.

The relatively small nuclei of the microgametocytes were similar in structure and in method of division to those of the schizonts. In microgametocytes approaching maturity the nuclei were located near the surface. In each nucleus there was a deeply staining band, possibly derived from the karyosome, around the outer margin of a hemisphere of lightly staining matter. The microgametes were apparently derived from this deeply staining band. Early microgametes were stubby rods; later stages were longer, more slender, and slightly curved. Microgametes were

not seen in fresh preparations. Mature microgametocytes were somewhat larger than macrogametocytes, but in the same range of size (Plate III, Fig. 21).

In macrogametocytes the nucleus was characterized by the presence of a relatively large spherical karyosome in an area clear except for some lightly staining matter at one side of the karyosome (Plate III, Fig. 19). The cytoplasmic granules were at first distributed at random, but in later stages there was a distinct layer of small granules near the surface, and a less distinct inner layer of larger granules (Fig. 20). As described for other species of *Eimeria* by Hosoda (1928), Roudabush (1937), and Lapage (1940), the outer layer stained red with Heidenhain's azan, while the inner layer stained yellow or orange. Lapage stated that the outer and inner layers of granules formed the outer and inner walls of the oöcyst, respectively, and this seems to be true also of *E. bovis*.

Penetration of the macrogametocyte by microgametes was not seen, but two stages in union of their respective nuclei were observed, one just before fusion (Plate III, Fig. 22), the other in the process of fusion (Fig. 23). In these specimens the oöcyst wall was incompletely formed.

The completed oöcyst usually showed a small number of fine granules (Plate III, Fig. 24), but sometimes no granules were visible. The nucleus had a homogeneous ground substance, staining a little more deeply than the less homogeneous cytoplasm. The usually eccentric karyosome was irregular in shape or sometimes divided into two or more parts, and was smaller than that in the macrogametocyte. The nuclear membrane was usually uneven in outline.

In most cases discharge of oöcysts began on the 18th to 20th day after inoculation. Usually considerable numbers of oöcysts were discharged during a period of 5 to 7 days, following which small numbers were present in the feces for 2 to 3 weeks. The symptoms and pathological changes have been described in detail elsewhere (Hammond, Davis, and Bowman, 1944). Diarrhea, usually beginning about the time the first oöcysts were discharged, was followed in severe infections by bloody diarrhea and tenesmus. The pathological changes, in most cases limited to the cecum and colon, included congestion, hemorrhage, destruction of the intestinal glands and of the mucosa, and formation of a membrane. The symptoms and pathological changes were directly associated with the sexual stages, rather than with the asexual stages, under the conditions of the present study.

DISCUSSION

Structures resembling the schizont stage of *E. bovis* have been reported from the intestine of cattle by a number of investigators. Smith (1893) gave an excellent description of bodies in bovine villi which appear to be identical with the schizonts of *E. bovis*. He described the merozoites, and stated that their movement was like that described by Eimer for coccidia in the intestine of the mouse. However, Smith concluded that the parasite does not belong to the coccidia, but has characters which might bring it within the range of the Sarcosporidia. Marotel (1907) reported finding similar bodies in the intestine of cattle, and suggested that they were the schizont stage of the species of coccidia found in cattle, *Eimeria zurnii*. Bugge (1910) found sharply circumscribed structures about the size of a pin-head in the intestine of a calf, and stated that they were the immature stages of either gregarines or coccidia.

Besnoit and Robin (1912) described a parasite of the skin of cattle, which was later found by Franco and Borges (1916) to occur also in the connective tissue and fasciae of the muscles. This parasite has been given the name of *Globidium besnoiti.* Some workers (Nöller, 1920; Henry and Masson, 1932) have concluded, without sufficient justification, however, that the cyst-like structure found in the intestine is identical with the cutaneous form, and thus have included it under the name *Globidium besnoiti.*

Müller (1914) found, in the intestine of healthy cattle, bodies ranging between 120 and 300 microns in length and containing many thousands of crescent-shaped structures. He considered these bodies to be schizonts of coccidia.

Railliet (1919), in discussing previous work on this subject, suggested that the bodies be called *Gastrocystis smithi,* and concluded that their relation with *E. zurnii* was not at all established. Bruce (1921) reported finding cyst-like bodies in the small intestine of a calf; one of these bodies was found to contain merozoites, and was therefore considered to be a schizont. Van Nederveen (1922) found globular bodies in sections from a calf's intestine which corresponded to the description of the schizont stage of *E. bovis* except that the merozoites were smaller (4 to 8 microns). The measurements were apparently made in sectioned material, which might account for the discrepancy in size. Van Nederveen referred to the structures as *Gastrocystis smithi* and stated that they do not represent the schizont stage of coccidia, nor belong to the Sarcosporidia. Pillers (1928) described bodies which he called *Globidium smithi* in the mucosa of the small intestine of a bull. He could find no support for the view that the bodies were coccidia, or Sarcosporidia. Hassan (1935) found cyst-like structures in both the ileum and the pyloric region of the abomasum of cattle in India. According to his description the cysts reached a diameter of 1 mm and the bodies within measured 13 by 2 to 2.5 microns. In the present study the maximum diameter of a schizont was found to be 435 microns, but the measurements given for the bodies within the cyst fall within the range found for merozoites. However, in the present study no schizonts of *E. bovis* were found in the abomasum. Hassan considered that the structure found by him was new, and he gave it the name *Globidium fusiformis.* Boughton (1942) found that the cyst-like bodies occurred in association with coccidial infections, especially those of *E. bovis,* and he observed a discharge of *E. bovis* oöcysts 5 days after feeding material containing the cyst-like structures to a week-old calf. On the basis of these observations he concluded that the cyst-like structures were schizonts of one or more species of bovine coccidia, *E. bovis* being the most likely in the material observed by him.

There is a possibility that some of the structures described as *Globidium* or *Gastrocystis* in the intestine of cattle are not identical with the schizont stage of *E. bovis* as described in the present paper. However, it seems logical to consider all of these, with the possible exception of the bodies found in the abomasum by Hassan (1935), as schizonts of *E. bovis,* until future work shows that more than one species of coccidia is involved.

It has been suggested that the so-called *Globidium* or *Gastrocystis* in some other hosts represents stages in the life history of *Eimeria* species. Moussu and Marotel (1902) advanced this idea for the form found in the small intestine of sheep; later workers have disagreed with their interpretation. Guyènot, Naville, and Ponse (1922) found what they considered to be an aberrant type of development of

Eimeria tropidonotus in the small intestine of the snake *Natrix tropidonotus,* in addition to stages typical of coccidian life history. These aberrant forms involved penetration of "special wandering cells" by merozoites, and growth into very large schizonts, accompanied by hypertrophy of the host cell and nucleus. Naville (1931) stated that he was tempted to include *Globidium* with the coccidia chiefly because of this finding in *Eimeria tropidonotus.* However, Harant and Cazal (1934) found similar large subepithelial structures in *Tropidonotus viprinus,* which they considered to be identical with the large schizont of Guyènot, Naville, and Ponse. Since no typical stages of *Eimeria* were present, they concluded that this structure belonged to the genus *Globidium,* and they named it *Globidium navillei.* Their views are that *Globidium* cannot be considered as belonging to *Eimeria* because of the large size and the number of merozoites each individual attains, but that it might be a connecting form between the coccidia and *Sarcocystis.*

Recently, a series of investigations by different workers has brought to light a pattern of development involving *Globidium,* and ending with *Eimeria*-type oöcysts in the camel (Henry and Masson, 1932; Enigk, 1934; and Naville, 1936), armadillo (Reichenow and Carini, 1937), and horse (Reichenow, 1940; Hemmert-Halswick, 1943). In these cases the *Globidium* stage is represented by the macrogametocyte and microgametocyte, and perhaps also by the schizont, although this stage has not yet been clearly worked out. The oöcysts are unusually large (about 75 microns long) and have a thick outer membrane around the oöcyst proper. Henry and Masson (1932) suggest that a certain group of species of *Globidium,* including *G. leuckarti* in the horse, *G. faurei* in the intestine of sheep, *G. cameli* in the camel, and *G. besnoiti* in the small intestine and skin of cattle, be defined as coccidia whose endogenous reproductive forms reach a large size, and which have large oöcysts with thick walls. The group was to be placed in the coccidia either near or in the genus *Eimeria.* It has been pointed out elsewhere in this paper that there is not sufficient reason for grouping together the skin and intestinal forms of the so-called *Globidium* in cattle. It is obvious that the life history of *E. bovis,* although it includes a *Globidium* stage, does not fit into the pattern of the forms found in the camel, horse, and armadillo. Work in progress at the U. S. Regional Animal Disease Research Laboratory has shown that in another species of bovine coccidia, *E. auburnensis,* the parasites in the sexual stage are located beneath the epithelium in the small intestine, a location similar to that described for the forms in the camel, armadillo, and horse, but information is lacking concerning the asexual stages of this species.

The asexual stage of the life history of *E. bovis* is different from the classical description of that of eimerian coccidia in parasitizing cells of tissue other than epithelial, in its location in the center of a villus, in the large size it attains, in the large numbers of merozoites produced by a single schizont, and in the occurrence of a single generation of schizonts. Schaudinn (1902) found that *Cyclospora caryolytica* occurred in connective tissues as well as in epithelial tissue but, so far as is known, no case has previously been described in which a stage of a coccidian organism is typically located in tissues other than epithelial. It appears logical that the occurrence of the schizont in the central lacteal provides a favorable environment for long continued growth, ending in a large schizont with so many merozoites that only one generation accomplishes a degree of multiplication similar to that of several generations in other *Eimeria.*

The question arises as to whether *E. bovis* should be kept in the genus *Eimeria* in the light of the radical departures in its asexual development from that of other *Eimeria*. It is the viewpoint of the authors that the question of the systematic position of *E. bovis* can be better taken up when the life histories of other species of coccidia in cattle, sheep, and other hosts have been worked out in detail.

The occurrence of a single asexual generation is in agreement with Boughton's (1943) results in treatment with sulfaguanidine of calves inoculated with *E. bovis*. He found that administration of the drug was effective against *E. bovis* when given during the week beginning 13 days after inoculation but was not effective when given earlier. The time at which the administration of the drug begins to be effective corresponds with the approximate time merozoites are released from the schizonts.

SUMMARY

1. A total of 51 calves were used in an experimental study of the life history of *Eimeria bovis*. Forty-nine calves were inoculated *per os* with sporulated oöcysts of *E. bovis*, killed at times ranging between 15 hours and 30 days later, and examined for stages of *E. bovis*. Two received inoculations of schizonts and merozoites directly into the intestine or into a Thiry's fistula. In 10 of the 49 calves enterectomies or other surgical operations were performed in association with the experimental infection. Daily fecal examination for oöcysts were made in all of these calves.

2. Sporozoites were found in the lower small intestine of calves killed 15, 17, 18, 30, and 46 hours after inoculation, but none were found in calves killed 21 and 24 hours after inoculation. In filtered intestinal fluid to which oöcysts were added before incubation, evidence of excystation was obtained in two of four trials. When oöcysts in a closed cellophane bag were left in a Thiry's fistula of a calf for 18 to 46 hours, sporocysts, but not sporozoites, were liberated in all of four trials.

3. In a number of calves killed from 2 to 4 days after inoculation no organisms in any stage were found. Early schizonts, found in calves killed 5 days after inoculation and later, closely resembled sporozoites in their nuclear structure, and in the possession of two extra-nuclear bodies staining intensely with iron-haematoxylin. This similarity together with the apparent absence of intermediate stages indicates that these schizonts arise directly from sporozoites.

4. The schizonts occurred in the posterior half of the small intestine, occupying cells in the lumens of central lacteals, or, in the earliest stages, in their walls. Early schizonts were seen in endothelial cells of the endothelial lining of lacteals, but there is a possibility that they may also parasitize other unidentified cells of the reticular connective tissue of the villus. The growth of the schizont stretches the cytoplasm of the host cell into a thin membrane. An additional outer membrane is formed from flattened cells.

5. The nucleus of the schizont undergoes repeated divisions, and in approximately half-grown schizonts the nuclei become arranged around the surface of small spheres. Merozoites are formed radially around these spheres.

6. Schizonts become macroscopic in size, and in this stage they have been previously described as *Globidium* or *Gastrocystis*. Mature schizonts, first seen 14 days after inoculation, were present in greatest numbers 16 to 18 days after inoculation. They averaged 281 by 203 microns, and contained an average of 120,000 merozoites. The average length of merozoites was 11.2 microns.

7. There was apparently only a single generation of schizonts. Injections of schizonts and merozoites into the Thiry's fistula, or into the ileum of a calf, were followed 4 or 5 days later by discharge of *E. bovis* oöcysts.

8. Occurrence of parasites in the sexual stage in most cases was limited to the cecum and colon. Seventeen days after inoculation was the earliest time that such parasites were found. Development of the microgametocyte and macrogametocyte is described. Discharge of oöcysts began in most cases on the 18th to 20th day after inoculation. Symptoms and pathological changes were directly associated with the sexual stages.

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Explanation of Plates

Photomicrographs of stages in the life cycle of *Eimeria bovis* from experimentally infected calves. Except where otherwise stated, all figures are of specimens in sections of tissue fixed in Zenker's fluid, and stained with iron-haematoxylin. Photographed by Leonard Reid Davis and B. T. Simms.

Plate I

Fig. 1. Sporulated oöcyst, somewhat flattened by pressure of cover slip, showing sporozoites in spores. ×1500.

Fig. 2. Spore released from oöcyst while in Thiry's fistula. ×1500.

Fig. 3. Early schizont, showing spherical body at left, karyosome of nucleus near center, and crescent shaped body at right. The host cell's nucleus is immediately above the schizont; its cytoplasm is indistinct. ×1200.

Fig. 4. Early schizont showing karyosomes of two nuclei, with spherical body toward center, and crescent-shaped body at right. Cytoplasm of host cell is not visible. ×1200.

Fig. 5. Early schizont, showing position along wall of central lacteal. Visible at top is nucleus of cell resembling endothelium adhering to host cell. Spherical body is at bottom of schizont. ×1500.

Fig. 6. Early schizont, with spherical and crescent-shaped bodies at upper left. Karyosomes of two nuclei are distinct, while those of three others are indistinct. ×1200.

Fig. 7. Later schizont, with nucleus of host cell at top left; crescent-shaped body, apparently extruded from schizont, lies in clear space between schizont and cytoplasm of host cell at top right. Many karyosomes of nuclei are visible in schizont. ×1200.

Fig. 8. Villus with four schizonts in various stages of development in central lacteal. ×300.

Fig. 9. Schizont showing early stage of arrangement of nuclei at the surface of small spheres. Nucleus of host cell is at lower right. ×300.

Plate II

Fig. 10. Villus with two schizonts, each showing the crescent-shaped body in clear space between schizont and cytoplasm of host cell. ×500.

Fig. 11. Schizont with nuclei arranged at surface of spheres. ×500.

Fig. 12. Merozoites from smear of crushed mature schizont, stained with Wright's and Giemsa's stains. ×1000.

Fig. 13. Portion of schizont approaching maturity, with merozoites arranged regularly. ×500.

Fig. 14. Portion of schizont invaded by large mononuclear cells and granulocytes. At upper right is a portion of an uninvaded schizont, with nucleus of a flattened cell in the membrane around the host cell. ×800.

Fig. 15. Mature schizont. A layer of the lamina propria lies between the schizont and the flattened epithelium of the villus. Clear space at lower left is probably an artifact. Nucleus of host cell is at bottom. ×200.

Fig. 16. Portion of mature schizont at tip of villus, showing merozoites. Fixed in Helly's fluid. ×1000.

Plate III

Fig. 17. Parasites in early sexual stages in epithelial cells at base of gland in cecum. Karyosomes are distinct. ×1500.

Fig. 18. Early microgametocyte (at bottom) and macrogametocytes. ×1500.

Fig. 19. Later macrogametocytes. Karyosome and cytoplasmic granules are especially distinct in specimen at upper left. ×1300.

Fig. 20. Macrogametocytes approaching maturity, with outer and inner layers of cytoplasmic granules. Fixed in Helly's fluid. ×1500.

Fig. 21. Microgametocytes with microgametes (middle left, bottom and lower right) and macrogametocytes (bottom left, upper right). ×1300.

Fig. 22. Stage before fusion of nucleus of macrogametocyte with nucleus of microgamete. ×1500.

Fig. 23. Nuclei in process of fusion. ×2000.

Fig. 24. Oöcyst in section of colon, with nucleus at center. ×1500.

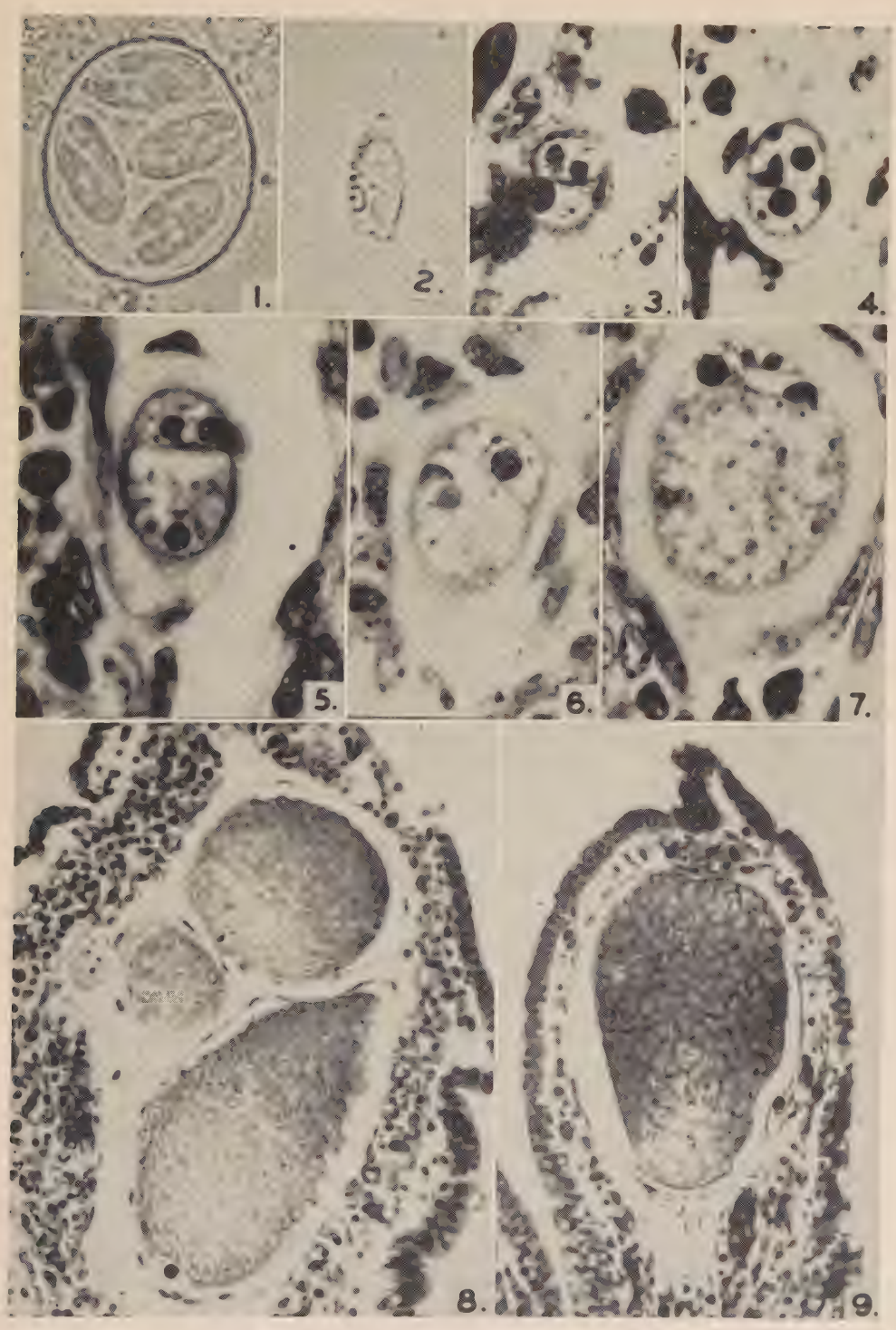

PLATE I

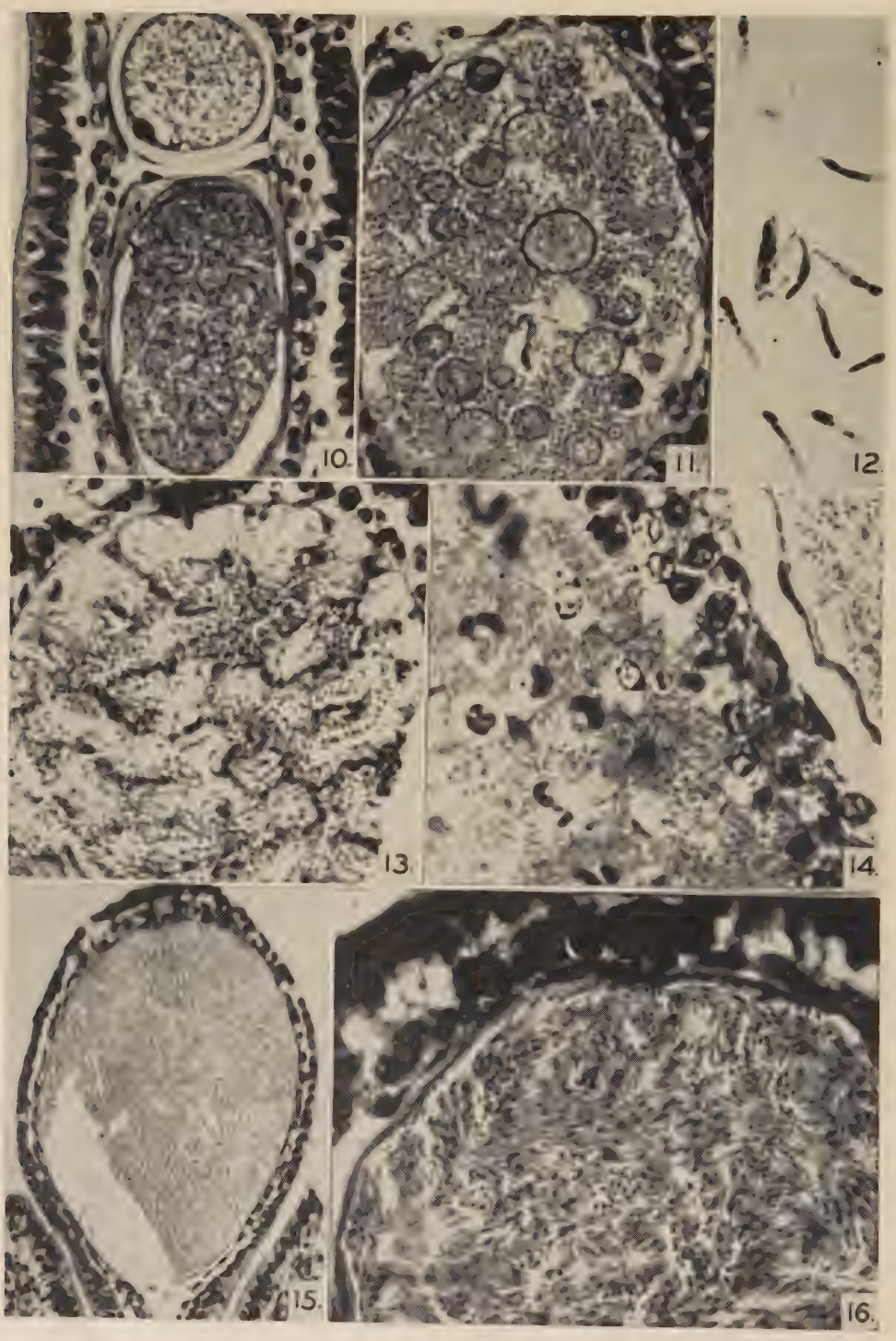

PLATE II

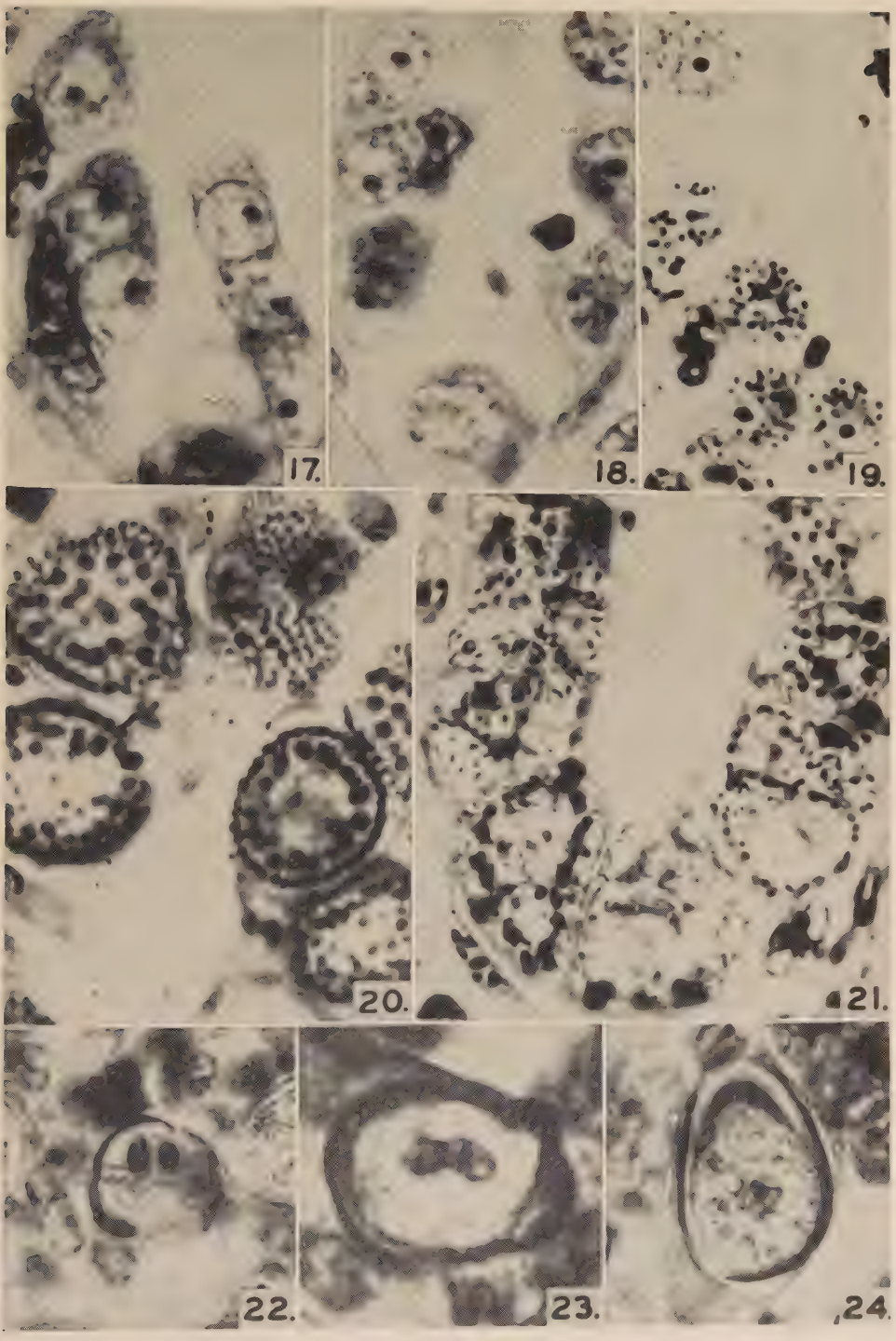

PLATE III

BENEDENIA NOBLEI, A NEW MONOGENETIC TREMATODE OF THE PACIFIC COAST ROCK FISH*

ROBERT J. MENZIES
College of the Pacific

A new species of monogenetic trematode collected on July 7, 1936, by Dr. Alden E. Noble at Monterey Bay, California, is described from the gills of the Pacific Coast Rock Fish. The specific determination of the host was made by Dr. Rolf L. Bolin of the Hopkins Marine Station.

The specimens were fixed in formalin-alcohol-acetic fluid and were preserved in 70 per cent alcohol. Whole mounts were stained in alum carmine; details of structure not readily observable on whole mounts were taken from frontal sections stained in alum haematoxylin and eosin.

The writer wishes to express appreciation for the aid of Dr. Alden E. Noble under whose direction this investigation was made.

Family CAPSALIDAE Baird, 1853
Subfamily BENEDENIINAE Johnston, 1931
Benedenia noblei n. sp.
(Figs. 1–5)

Description: Body is 1.662 to 1.905 mm long by 0.794 to 0.932 mm in greatest width, lateral borders taper anteriorly until they reach anterior haptor where they project laterally as the antero-lateral borders of haptor. The extreme anterior haptoral margin of body is slightly convex while the posterior end, exclusive of posterior haptor, is slightly concave. No integumental spines or papillae were observed. Anterior haptor consists of two ventrally placed suckers located at a 45 degree angle to main body axis. Left anterior sucker measures 0.170 to 0.202 mm long by 0.121 to 0.157 mm wide. Anterior half of each sucker is glandular in nature and sharply differentiated from posterior half by antero-lateral limits of its marginal membrane. Posterior haptor is stalked, ventrally placed and when in a contracted state typically divided into a large oval anterior portion and a smaller postero-median lobe. A series of square segments measuring 0.032 mm wide constitute the marginal membrane of posterior haptor. Posterior haptor measures 0.502 to 0.772 mm at greatest width and contains three pairs of hooks: a spindle shaped anterior pair measuring 0.079 to 0.103 mm long by 0.027 to 0.028 mm wide, a narrow elongate middle pair measuring 0.085 to 0.086 mm long by 0.005 to 0.007 mm wide, and a large blade-like posterior pair measuring 0.086 to 0.116 mm long by 0.026 to 0.045 mm wide. Mouth is ventrally situated, 0.054 mm long by 0.072 mm wide and is continuous with ovate pharynx which measures 0.162 to 0.203 mm long by 0.138 to 0.194 mm wide. A conspicuous salivary cell aggregate surrounds postero-laterally the juncture of right and left digestive ceca. A short esophagus joins digestive ceca caudad to pharynx. Digestive ceca are branched and asymmetrical, the right branch being more highly arched than the left. Numerous secondary branches, a few of which bifurcate, are given off from the outer margin of digestive ceca. Genital pore opens into ventro-lateral depression posterior to left sucker of anterior haptor. Genital atrium is large, formed by union of neck of cirrus pouch with uterus at a point immediately anterior to first caudal turn of left cecum. Two testes measuring 0.340 to 0.404 mm long by 0.227 to 0.261 mm wide are located in posterior half of body. Their medial borders touch one another and their lateral borders are overlapped ventrally by postero-lateral vitelline ducts. Vas deferens, large and highly convoluted, enters neck of cirrus pouch anterior to apex of oötype. At caudal end of cirrus pouch it receives a small duct from prostatic reservoir before it continues anteriorly as cirrus proper. Prostatic reservoir ovoid and located within caudal end of cirrus pouch. Ovary is 0.186 to 0.251 mm long by 0.156 to 0.178 mm wide, located between vitelline reservoir and testes. It is divided internally into two large chambers, a right seminal receptacle and a left fertilization chamber. Both divisions are of similar size. Oviduct leaves dorsal surface of ovary at a point near right antero-

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* Contribution from the Parasitological Laboratory, College of the Pacific, Stockton, California.

lateral ovarian margin and after receiving a short duct from vitelline reservoir proceeds anteriorly to oötype. Shell glands completely surround oötype and antero-posteriorly occupy majority of space between pharynx and vitelline reservoir; laterally they occupy the space between cirrus pouch and vagina. A pyramidal egg, measuring 0.162 mm long by 0.129 mm wide, is present in the oötype of the type specimen and presents a long coiled filament at its base. Vagina consists of three portions, a distal tubular portion which opens to exterior dorsal to opening of genital pore, a middle swollen seminal receptacle, and a proximal tubular portion which connects with left anterior apex of vitelline reservoir. A large triangular vitelline reservoir is present and is located in middle one half of body to left of midline. It connects laterally on each side with horizontal vitelline ducts formed by union of the four main lateral vitelline ducts. Vitelline follicles are large and measure 0.032 mm in average diameter. They follow the body margin laterally and cover the ceca medially except for a single characteristic rosette arrangement of follicles located within the arch of right cecal branch. Glands of Goto are located posterior to testes and consist of two semiovate bodies each of which contains 5–7 giant nuclei. The nuclei in turn contain three dark-staining rod-like structures. Among vitelline follicles and adjacent to outer margins of right and left ceca are located two excretory reservoirs whose excretory pores were not seen.

This description is based upon six specimens.

Host: Sebastodes paucispinus (Ayres, 1854).

Location: Gills.

Locality: Monterey Bay, California.

Type: U. S. Nat. Mus. Helm. Coll. (No number yet assigned.)

Comparisons.—The species while agreeing closely with the generic diagnosis of *Benedenia* seems specifically quite distinct from any previously described member of the genus. If it can be said that a closer resemblance exists between *B. noblei* n. sp. and any other member of the genus, the author would be of the opinion that it was with *B. convoluta* Yamaguti, 1937. The two species are readily distinguished from one another, however, by a comparison of the following characteristics present in *B. noblei* n. sp. and absent in *B. convoluta:* (1) The rosette appearance of vitelline follicles within arch of right cecal branch; (2) the high curvature of neck of cirrus pouch; (3) the presence of both an intraovarian and a vaginal seminal receptacle; (4) the presence of an intraovarian fertilization chamber; (5) the presence of Glands of Goto; (6) the ratio of length of testes to body length (1 : 5 in *B. noblei* n. sp. and 1 : 8 in *B. convoluta*).

Reference to the above characteristics together with the comparison of haptoral hooks will serve to distinguish *B. noblei* n. sp. from all other species of *Benedenia* described to date.

SUMMARY

A new species of monogenetic trematode *Benedenia noblei,* is described from the gills of the marine fish, *Sebastodes paucispinus,* from Monterey Bay, California.

REFERENCE

YAMAGUTI, S. 1937 Studies on the helminth fauna of Japan. Part 19. Fourteen new ectoparasitic trematodes of fishes. (Published by author.) Lab. of Parasitol., Kyoto Imp. Univ.: 1–28.

EXPLANATION OF PLATE

All drawings were made with the aid of a camera lucida. Abbreviations are as follows:

A genital atrium
B salivary cell aggregate
C excretory reservoir
D vaginal seminal receptacle
E vas deferens
F ovarian fertilization chamber
G left branch of digestive cecum
H left Gland of Goto
I rosette arrangement of vitelline follicles
J vitelline follicle
K prostatic reservoir
L vitelline reservoir
M ovarian seminal receptacle
N right postero-lateral branch of vitelline duct
O right testes

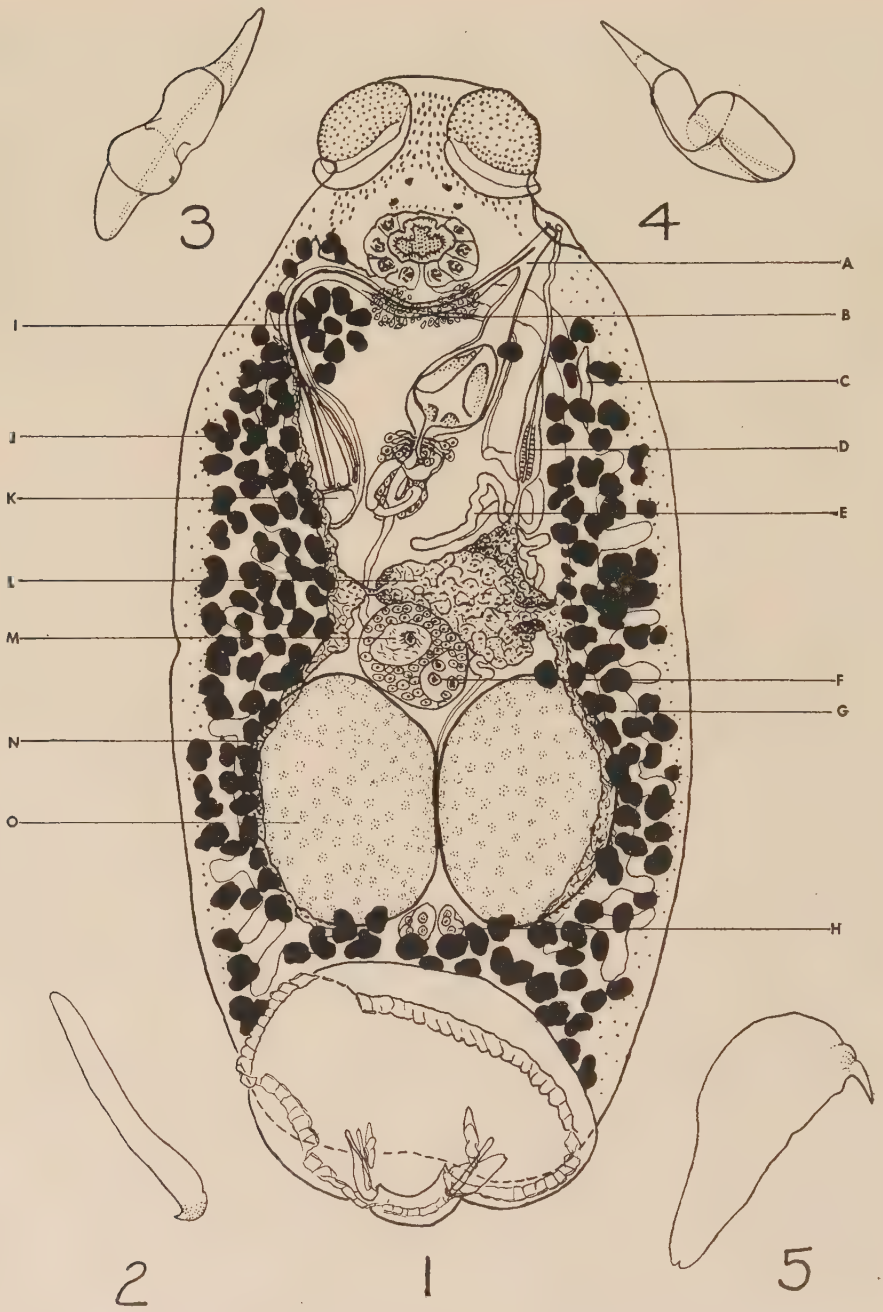
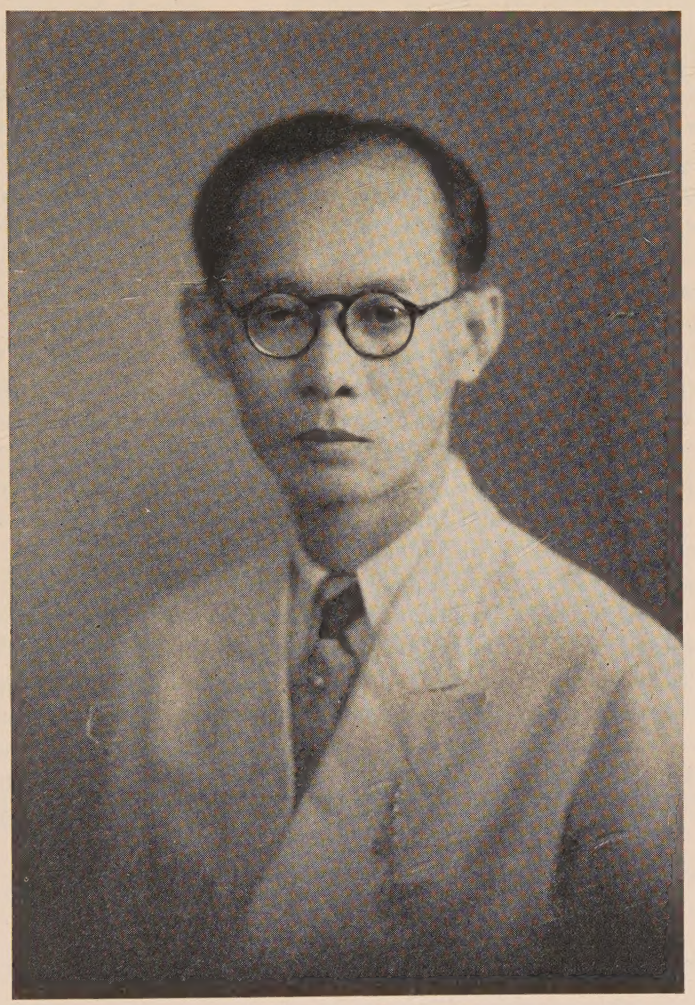


FIG. 1. *Benedenia noblei* n. sp., ventral view, 15×
FIG. 2. Left middle hook of posterior haptor, lateral view, 111×.
FIG. 3. Left anterior hook of posterior haptor, ventral view, 111×.
FIG. 4. Left anterior hook of posterior haptor, ventro-lateral view, from paratype, 111×.
FIG. 5. Left posterior hook of posterior haptor, lateral view, 111×.

IN MEMORIAM

Candido M. Africa (1894–1945)

In the death of Dr. Candido M. Africa, the Philippines lost a distinguished citizen, an eminent scientist and an inspiring teacher; his associates and colleagues lost a thoughtful and considerate friend. Born at Lipa, Batangas, Philippine Islands, on October 3, 1894, Dr. Africa was granted the M.D. degree from the College of Medicine, University of the Philippines, in 1920. As a fellow of the University of the Philippines in 1927 and later as a fellow of the Rockefeller Foundation, he studied



at the London School of Tropical Medicine, from which he received the degree of D.T.M. and H. in 1928. Later, he worked for some months in the laboratory of Dr. Fülleborn at the Institute für Schiffs- und Tropenkrankeiten at Hamburg before going to the United States where he was a guest investigator in the Department of

Helminthology, School of Hygiene and Public Health, The Johns Hopkins University, and in the Zoological Division of the Bureau of Animal Industry in Washington.

Upon his return to the Philippines, he was engaged in research at the Institute of Hygiene, in which he was appointed Associate Professor of Parasitology in 1931. Concurrently he held a similar position in the College of Medicine, University of the Philippines, with which the Institute is associated. In 1939, Dr. Africa was made a full professor. In the same year, he was selected as an official delegate of the Commonwealth of the Philippines to attend the 3rd International Congress of Microbiology in New York. En route, he visited various scientific laboratories in Italy, Hungary, Germany, Sweden, France and England. In April 1944, Dr. Africa was one of the candidates selected by the "Philippine Republic" to attend the Japanese sponsored medical conference held in Nanking.

Dr. Africa's parasitological interests have covered a wide range but his principal contribution concerned heterophyidiasis in man. As a consequence of his work it was demonstrated that these flukes, which were formerly believed to be limited exclusively to the intestine, have important additional pathological significance in man. Heterophyid eggs were found in lesions in various organs, including the heart, brain and spinal cord. Cardiac heterophyidiasis is a recognizable clinical entity in endemic areas. Other parasitological studies were concerned with *Bertiella studeri, Raillietina garrisoni* and *R. madagascariensis* as well as *Strongyloides stercoralis* and *Gnathostoma spinigerum.* He was one of the first workers to use the nematode, *Nippostrongylus muris,* in studying the problem of helminth immunity. Following his visit with Prof. A. Missiroli in Italy in 1939, he became interested in the problem of the exo-erythrocytic cycle in malaria employing *Plasmodium gallinaceum* in chicks. Between 1942 and 1945, he studied numerous brains from patients who died from malignant tertian malaria and was able to demonstrate that exo-erythrocytic stages occurred in infections with *P. falciparum.* Only a brief report of this work had been made at the time of his death and, unfortunately, his specimens and manuscripts were lost in the debris of the demolished building of the Institute of Hygiene.

As a guest of the Institute of Hygiene, University of the Philippines, I was with Dr. Africa for three years. During this time, I was greatly impressed by his keen interest in research and his ability and determination to carry on with his work in the face of discouragement and great difficulties, his admirable enthusiasm in teaching, his gracious personality and his kindness toward those with whom he worked.

Dr. Africa was killed at the door of his residence in Manila on February 12, 1945, at five o'clock in the afternoon, a short time prior to the entry of American troops into that portion of the city. He is survived by his widow and three children.

H. F. Hsü

RESEARCH NOTE

A TECHNIQUE FOR XENODIAGNOSIS OF FILARIASIS

Feeding experiments with reared mosquitoes were performed on an American marine infected with *Wuchereria bancrofti* in order to determine if microfilariae would more likely be detected in blood from the mosquito's stomach than in a sample examined directly from the patient. Earlier workers had reported that microfilariae were proportionately more numerous in blood ingested by the mosquito than in the peripheral capillary circulation of the patient. An experiment was devised to test this observation quantitatively.

In order to compare the volume of blood from the engorged mosquito's stomach with an equivalent volume of a sample from the host's circulation, the Electrohemometer, a colorimetric hemoglobinometer, was used. With this photo-electric instrument the amount of hemoglobin per unit volume of blood was measured.

A sample of whole blood was hemolysed by volumetric dilution with N/10 HCl in apparatus which was a standard part of the equipment of the Electrohemometer. Samples of the hemolysed blood were mounted in the instrument and readings made in terms of grams of hemoglobin per 100 cc. This reading (R) provided a standard against which later dilutions could be compared.

The dilution of any volume (V) of hemolysed blood can be determined by readings (r) provided the dilution is kept within the range of sensitivity of the instrument.

The microfilariae in a blood sample were recovered after hemolysis with N/10 HCl, centrifuging, and removal of the supernatant with a capillary pipette. The microfilariae were removed from the bottom of the tube with a capillary pipette, mounted under a cover-slip and counted (N) under the compound microscope. A calculation was made to determine the number of microfilariae per unit volume, 1 cc.

$$\frac{1}{V} \times \frac{R}{r} \times N = \text{Number of microfilariae per cc.}$$

when V = the final volume (cc) of the diluted hemolysed blood
R = grams of hemoglobin per 100 cc of host's blood
r = grams of hemoglobin per 100 cc of diluted hemolysed blood (a sample of V)
N = the number of microfilariae recovered from V.

By use of this formula a greater sample of blood can be used for microfilaria count.

Similarly the microfilariae from the blood-content of the mosquito's stomach were recovered and counted after the stomachs had been removed, the blood-content hemolysed, volume determined and readings made of the hemoglobin.

Mosquitoes used for this experiment were *Culex fatigans* which were collected in the larval and pupal stages and brought to the laboratory for emergence. A colony of *Aedes aegypti* reared in the laboratory was also used. The former species were larger and contained more blood when fully engorged. The latter species were smaller, took less blood when feeding, but had more dependable appetites. After removal of the engorged stomachs the head, thorax and abdomen were examined for microfilariae which may have migrated into the body cavity and thoracic muscles.

With the helpful cooperation of the patient, mosquitoes were fed on four occasions:

Experiment 1. March 29, 1944

In the first feeding experiment 6 *C. fatigans* which had fed on the host between 9:00 and 9:30 PM yielded 4 worms.

Experiment 2. April 11, 1944

From 15 *A. aegypti* which engorged between 3:00 and 4:00 PM 8 microfilariae were recovered.

Experiment 3. April 13, 1944

From 15 *A. aegypti* which engorged between 8:00 and 9:00 PM 19 microfilariae were recovered.

At the beginning of each of these experiments a sample of the host's blood was taken for subsequent determination of the hemoglobin content (R) and the number of microfilariae per cc.

Calculations revealed that in each instance the yield of worms from the mosquitoes was greater than the number which were found associated with an equivalent amount of hemoglobin in the patient's peripheral capillary circulation. The ratios determined in the 3 experiments were respectively 7.4 to 1, 9.1 to 1, and 13.3 to 1.

The above experiments show that the microfilariae of *Wuchereria bancrofti* were more numerous per unit volume of blood within the stomach of the mosquito than they were in the patient's peripheral capillary circulation.

This method of xenodiagnosis may be used in a laboratory where mosquito colonies are available and a technician skilled in mosquito dissections is at hand. Average time per dissection (removal of stomach) was 2.5 minutes (29 dissections in 75 minutes). It may prove to be useful in light infections of man just as it proved useful in light infections in the course of transmission experiments of *Dirofilaria scapiceps* from snowshoe hares to domestic rabbits (Highby, 1943, J. Parasitol. **29**: 253).

Experiment 4

Ten specimens of *A. aegypti* which engorged between 3:30 and 4:00 PM March 29, 1944, were dissected after 4 days storage in the refrigerator. Examination revealed a total of thirteen microfilariae from the ten mosquitoes. Two mosquitoes had 1 worm each in the thorax, four had 1 worm each in the stomach, one had 6 worms in the stomach and 1 in the thorax, and three had none. This distribution illustrates that several mosquitoes should be used on a light infection.

This work was accomplished at the 8th Medical Laboratory, APO 923, Brisbane, Australia. The patient had been exposed in British Samoa. Access to the patient was afforded by the 105th General Hospital, U. S. Army and Mobile Hospital Unit No. 9, U. S. Navy.—Capt. Paul R. Highby, *Sanitary Corps, AUS, 2082 Como Ave., St. Paul 8, Minnesota.*